COMBINED ENZYMATIC HYDROLYSIS AND FERMENTATION OF ASPENWOOD USING ENZYMES DERIVED FROM *Trichoderma harzianum E58*

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In memoria of Henri Normand who died during
the project. His ready smile and
joie de vivre are missed by all.

List of abbreviations

SHF - simultaneous hydrolysis and fermentation

CHF - combined hydrolysis and fermentation

SEA-WS - steamed aspenwood - water solubles

SEA-WI - steamed aspenwood - water insolubles

SEA-WAI - steamed aspenwood - water and alkali insolubles

FPU - IU of filter paper activity

MBTU- million British Thermal Units

ODT- oven dried ton

Preface

This report presents the significant results of the work performed for the project entitled "Combined hydrolysis and fermentation of lignocellulosic residues", generously financed by Energy Mines and Resources Canada.

The project goal was to study the conversion of aspenwood to liquid fuels such as ethanol, butanol or butanediol. The process was based on the enzymatic hydrolysis of the wood cellulose and hemicellulose and the subsequent fermentation of the sugar to liquid fuels. This study will advance the knowledge base in the bioconversion field and provide much needed data for the eventual commercialization of the process.

The work described in this report was performed during the years 1985-87. The report is intended for an audience interested in the commercialization of this work. The report contains the published technical data generated by the research and would be of interest to researchers in the bioenergy field.

TABLE OF CONTENTS

page

I EXECUTIVE SUMMARY vi

II INTRODUCTION 1
- 2.1 Background 1
- 2.2 Background on _T. harzianum_ E58 8
- 2.3 Cellulose conversion 9
- 2.4 Techno-economic state of the art, 1984 12

III FACTORS LIMITING HYDROLYSIS 14

Substrate effects limiting the efficiency of hydrolysis 15

Use of _Trichoderma harzianum_ E58 cellulases to determine optimal enzyme concentration for hydrolysis of Solka Floc BW 300 19

IV RECYCLE- ENZYMES AND CELLULOSE 42

Recycle of enzymes and substrate following enzymatic hydrolysis of steam-pretreated aspenwood 43

Enzymatic conversion of lignocellulosic material to fuels 51

Influence of growth substrate on production of cellulase enzymes by _Trichoderma harzianum_ E58 55

Recycle of cellulase and lignocellulosic residue after hydrolysis of steam-treated aspenwood 61

V FERMENTATION OF HEMICELLULOSE 85

A simple method for xylanase preparation used for the hydrolysis and fermentation of hemicellulose to butanediol 86

Comparative fermentability of enzymatic and acid hydrolysis of steam-pretreated aspenwood hemicellulose by _Pichia stipitis_ CBS 5776 93

Effect of initial glucose:xylose ratio and total substrate concentration on xylose fermentation by _Pichia stipitis_ CBS 5776 112

VI MARKET STUDY FOR ETHANOL, BUTANOL AND BUTANEDIOL 118

VII DISCUSSION 121

REFERENCES 128

LIST OF FORINTEK CANADA CORP. PUBLISHED PAPERS ON BIOCONVERSION

LIST OF FORINTEK CANADA CORP. REPORTS ON BIOCONVERSION

I. EXECUTIVE SUMMARY

The goal of the project was to study the conversion of aspenwood to ethanol, butanol or butanediol. The conversion was based on steam explosion as the pretreatment method and enzymatic hydrolysis of the carbohydrate polymers, cellulose and hemicellulose. The enzyme system used was derived from the fungus *Trichoderma harzianum* E58. The strain used was a wild type that had not been mutated or genetically manipulated. This organism was chosen because it produced a cellulase system that could degrade crystalline cellulose to glucose. The fungus produced high levels of β-glucosidase which made it an attractive organism for further study. *T. harzianum* also produced high levels of xylanase enzymes that were capable of hydrolysing hardwood hemicellulose to xylose.

The aspenwood was steamed at 240°C for 80 seconds and then water and alkali extracted. The insoluble residue was 84% cellulose and was used for both enzyme production and production of glucose. The glucose produced was fermented to ethanol.

The water-soluble fraction (SEA-WS) was primarily acetylxylan, which was further hydrolysed to D-xylose and acetic acid. This fraction also contained lesser amounts of other fermentable sugars such as glucose and galactose and their uronic acids. Both lignin- and sugar-degradation products formed during the steam-pretreatment were present in the water-soluble fraction. These products, together with acetic acid, acted synergistically to inhibit fermentation.

Before fermentation was possible, the acetylxylan had to be hydrolysed and the inhibitors removed. Hydrolysis was done using either acid or enzymes. Enzymatic hydrolysis, using xylanases produced by *T. harzianum,* was found to generate less fermentation inhibitors compared with sulphuric acid hydrolysis. Acetic acid appeared to be the major inhibitor when present at concentrations above 1 g/L, however, the removal of lignin-derived inhibitors by solvent extraction was found to be necessary for complete fermentation at concentrations around 5% (w/v) total solids.

The hemicellulosic sugars could be fermented by three processes. The production of 2,3-butanediol by the bacterium, *Klebsiella pneumoniae* was the subject of previous studies at Forintek and was found to be applicable to SEA-WS fermentation following inhibitor removal treatments. Unfortunately, a market study on industrial solvents and liquid fuels found no available market for 2,3-butanediol produced from biomass. Work on this fermentation was subsequently stopped in favour of a more marketable product.
The production of acetone, butanol and ethanol (ABE) from the fermentation of hemicellulose sugars by the anaerobic bacterium, *Clostridium acetobutylicum* has been demonstrated at Forintek. Some initial ABE fermentation optimization studies were carried out in the present study, however, the market for ethanol

was more defined than for the ABE fermentation products. Consequently, our fermentation research centred on the production of ethanol from hemicellulose.

The yeast, *Pichia stipitis*, produced near theoretical ethanol yields from the hemicellulose hydrolysate only after removal of the fermentation inhibitors mentioned earlier. Acetic acid, at concentrations above 1 g/L, was the main inhibitor of xylose fermentation by *P. stipitis*. An evaporation treatment could remove the majority of the acetic acid, but extraction with ethyl acetate was necessary for complete removal of the fermentation inhibitors.

The role of lignin in the process was investigated. Although lignin had no effect on hydrolysis there were several advantages to removing lignin prior to enzyme production and cellulose hydrolysis. The lignin in the substrate increased the bulk to be handled at each stage. The steamed material had a low bulk density which caused handling problems at high substrate concentrations. Lignin, in combination with small amounts of cellulose, was found to strongly adsorb the cellulase enzymes. Appreciable amounts of cellulase enzymes remained absorbed to the hydrolysis residue and were unavailable for recycle. The soluble breakdown products of lignin were found to be inhibitory to the cellulase complex of *T. harzianum*. These products were also inhibitory to the enzyme production phase.

Cellulose hydrolysis has an initial rapid rate, which levels off drastically after about 8 hours. The structural limitations imposed by the physical characteristics of cellulose and the change in structure as hydrolysis proceeded were found to be the most significant factors limiting hydrolysis. It was suggested that the most promising area for research would be the treatment of aspenwood prior to or during enzymatic hdyrolysis.

The need to divert part of the cellulose to enzyme production reduced the potential glucose yield. Various ways of minimizing the amount of cellulose needed for enzyme production were investigated. Attempts to increase enzyme productivity or yield using fed-batch fermentations were not successful. The use of lactose for enzyme production by *T. harzianum* resulted in lower yield and quality of cellulase enzymes. The physical characteristics of the cellulose were found to have an effect on the cellulase enzyme profile produced by *T. harzianum*.

The unhydrolyzed substrate from the cellulose hydrolysis was found to be an excellent substrate for production of highly active cellulase complex. This material had to be used in combination with fresh cellulose since the presence of soluble lignin products inhibited production.

After cellulose hydrolysis, very little enzyme activity remained in the supernatant portion of the reaction mixture. The majority of the cellulase enzymes were adsorbed to the residue and thus were unavailable for recycle. Even under conditions of high conversion of cellulose, the residue still adsorbed significant quantities of enzymes. It was suggested that research in this area focus on means of desorbing the enzymes from the hydrolysis residue.

None of the strategies used to decrease the amount of cellulose required for enzyme production were successful and we concluded that *T. harzianum* E58 was not suitable for use in a commercial bioconversion process.

The scientific knowldege base in the bioconversion field was advanced by the work performed under this contract. The contract resulted in the publication of nine articles on the subject of limitations to cellulose hydrolysis, induction of cellulase enzymes, hemicellulose utilization, recycle of cellulase enzymes and recycle of residual cellulose.

II. INTRODUCTION

2.1 Background

The major goal of this project was to study the conversion of aspenwood to ethanol, butanol or butanediol. Wood has a complex structure, being composed of three biopolymers: Cellulose, a glucose polymer, hemicellulose, a pentose polymer, and lignin, a phenolic polymer. Cellulose can be hydrolysed to glucose using either acid or enzyme catalysts. The enzymatic hydrolysis of cellulose has the advantage of high theoretical yields, lower temperature requirements and low environmental impact.

The efficient utilization of aspenwood requires that most or all of the components be converted to useful products. Research in the utilization of the total carbohydrates (cellulose as well as hemicellulose) is therefore crucial in establishing an efficient bioconversion process.

The steam explosion process has been demonstrated to be effective in separating the wood into cellulose, hemicellulose, and lignin. After a simple water-extraction of the steam-exploded wood, the hemicellulosic carbohydrates (and some lignin) are generally present in the water-soluble fraction, leaving cellulose and lignin in the water-insoluble residue.

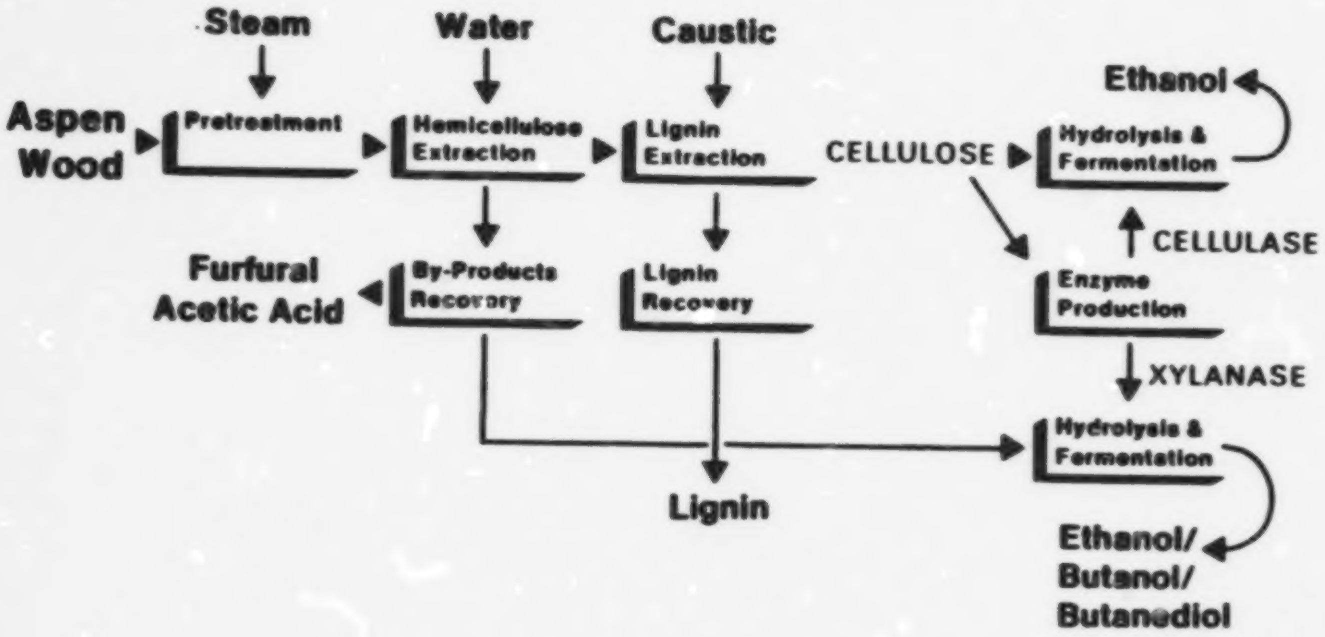
Conversion of aspenwood to solvents has three discrete steps involving pretreatment of the lignocellulosic substrate, enzyme production and combined hydrolysis and fermentation (CHF). There exists considerable interdependence between these three steps. Both the structure of the substrate and the enzyme system employed play major roles in limiting the application of this technology to a commercial process. The pretreatment process used in this project was steam explosion and the enzyme system used was the cellulase complex produced by the fungus *Trichoderma harzianum* E58.

A generalized process design based on steam pretreatment and enzymatic hydrolysis is presented in Figure 1. The various steps in the overall process are outlined below.

Steam pretreatment

Aspenwood must be pretreated prior to enzymatic hydrolysis because cellulose in native wood is largely inaccessible to enzymatic attack. Steam pretreatment has been shown to be an excellent pretreatment process to enhance enzymatic hydrolysis (Saddler and Brownell, 1982). There exists a close relationship between the extent and rate of enzymatic hydrolysis of cellulose and the steaming conditions used (Forintek Canada Corp., 1984). Steaming conditions affect separation of the three components; cellulose, hemicellulose and lignin. Steam pretreatment causes destruction of pentosan material so

Figure 1. **Simplified Flow Diagram of the Process Development Unit**


Steam
Water
Caustic
Aspen Wood
Pretreatment
Hemicellulose Extraction
Lignin Extraction
CELLULOSE
Hydrolysis & Fermentation
Ethanol
CELLULASE
Enzyme Production
XYLANASE
By-Products Recovery
Lignin Recovery
Furfural Acetic Acid
Hydrolysis & Fermentation
Lignin
Ethanol/ Butanol/ Butanediol

steaming times and temperatures must be optimized for pentosan solubilization while minimizing pentosan destruction (Forintek Canada Corp., 1983).

Based on previous ENFOR-funded research, we chose a steaming condition (240°C for 80 seconds) that would satisfy three criteria; cellulose accessibility, high pentosan solubility and low pentosan destruction. Research in pretreatment continued to investigate several steaming options involving high and low steam temperatures, acid catalysts and post-steaming treatments. Because pretreatment has a major impact on all other downstream processes, it is important to continually compare the pretreatment options with the selected base case.

Extraction processes

After pretreatment, wood chips are extracted and water-soluble hemicellulose and caustic-soluble lignin are obtained. This step was briefly investigated but was beyond the scope of the present project. The major requirement in this step is for extraction of material at high concentration to minimize costs.

Fermentation of water-soluble material

Water-soluble material (ie. hemicellulose) is hydrolysed to monomeric sugars and fermented to either butanediol, butanol or ethanol. Sugars in this stream are mostly five carbon sugars that cannot be fermented to ethanol using traditional fermentation yeasts. In recent years, novel yeasts have been discovered that can ferment five carbon sugars to ethanol. Bacteria such as *Klebsiella pneumoniae* or *Clostridium acetobutylicum* can ferment the five carbon sugars to either butanediol or butanol, respectively.

The major problem facing the utilization of water solubles is the presence of fermentation inhibitors. These inhibitors are produced during the steaming step and severely limit the ability of microorganisms to utilize the sugars and to produce solvents (Yu *et al.*, 1982; Mes-Hartree *et al.*, 1983; Nishikawa *et al*, 1988).

Production of butanediol from wood hydrolyzates

The products of hydrolysis of cellulose are basically glucose (and cellobiose) whereas a wide variety of products are obtained from hemicellulose hydrolysis, including sugars (xylose, arabinose, glucose, galactose, mannose, and their respective oligomers), uronic acids (glucoronic and galacturonic acids) as well as acetate (derived from the acetyl groups). To maximize the overall efficiency in the bioconversion process, we have screened for microorganisms with the ability to utilize all, or most, of these substrates for the production of fuels and chemicals.

Our recent work with *Klebsiella pneumoniae* (formerly *Aerobacter aerogenes*) strongly suggests that it is an ideal organism that can efficiently utilize both cellulose and hemicellulose hydrolyzates (Yu *et al*, 1982; Yu *et al* 1984 a,b; Yu *et al*, 1985). As this organism is nutritionally versatile it can efficiently utilize all the major sugars and uronic acids known to be present in wood hydrolysates and convert them to butanediol (and ethanol) at an efficiency approaching the theoretical maximum.

The major fermentation product of *K. pneumoniae* is *meso*-2,3-butanediol, however, low levels of ethanol, acetic acid, acetyl methyl carbinol (acetoin) and diacetyl (butanedione) are also produced together with gaseous products (hydrogen and carbon dioxide). The potential uses of butanediol are extensive and earlier workers, in pre-petrochemical times, proposed building a chemical industry based upon this compound. Butanediol can be readily converted to butadiene, a major constituent of synthetic rubber or can be dehydrated to give methyl ethyl ketone, an excellent solvent for glyptal, vinyl resins and lacquers. It can also yield isomers of octane to be used as fuel additive (octane booster) or be blended to give high grade aviation fuel. Recent studies have further extended the potential applications of butanediol as a chemical feedstock in various industries, including the synthesis of plastic (polyesters). In short butanediol can displace petroleum products if the technology is advanced enough to make the process more economical.

One disadvantage in the butanediol fermentation lies in the cost for product recovery. Due to the high boiling point of butanediol (180°C), the extraction of the solvents by conventional distillation is energy intensive and costly. A final solvent level of 8-10% (w/v) is desirable if product recovery is to be economical. By optimizing the culture conditions and by using adaptation, acclimatization, and double fed-batch fermentation, we are now able to achieve final butanediol concentrations of 80 g/L and 106 g/L from xylose and glucose, respectively.

Production of butanol from wood hydrolyzates

Alternatively, sugars derived from wood cellulose and wood hemicellulose could be used as substrates for acetone-butanol-ethanol (ABE) fermentation by *Clostridium acetobutylicum* (Mes-Hartree and Saddler, 1982). The major products are butanol, acetone, and ethanol (normally at a ratio of 6:3:1), but depending on the culture conditions, acetic and butyric acids can also become significant products.

Interest in ABE fermentation has increased as a result of the projected significance of butanol as liquid fuel and chemical feedstock. Butanol has a heating value of 29 MJ/L, which compares favourably with gasoline (31), ethanol (24), butanediol (27), and acetone (26). It also has the additional advantages of being non-explosive, water-tolerant (i.e., no phase separation with water), and having a high octane number.

Butanol can be readily converted to butadiene for the manufacture of synthetic rubber, and to butyl acetate, an important industrial solvent. The major by-product, acetone, can also be used as an industrial solvent (e.g., in the explosive industries). The gases formed are predominantly hydrogen and carbon dioxide and could be used for the production of methanol at an industrial scale. Finally the residues of the fermentation process are known to be rich in Vitamin B-12 and of value as animal feed supplement. The major handicap at present appears to be the economic competitiveness of the process with the petrochemical industries. The establishment of a simple process making use of an inexpensive source of fermentable carbohydrates (such as wood residues) could likely result in an economically viable process.

One major problem in ABE fermentation is the toxicity of the end-products to the fermenting organisms. Butanol appears to be the most potent inhibitor, but other products, including acetone, butyric acid, and acetic acid, can also accumulate to inhibitory levels during the process. Butanol by itself is toxic at low concentration (around 1.2%), and is known to act synergistically with other inhibitors as well. At present, all attempts to acclimatize the organism to butanol, either by serial subculturing or by selection through continuous cultivation, has uniformly been unsuccessful. Selection of mutants with increased resistance to butanol has also met with only marginal improvement in the fermentation process. It is therefore important that novel fermentation approaches be contemplated to overcome or circumvent this problem.

Production of ethanol from wood hydrolyzates

Ethanol production from hexoses such as glucose, mannose and galactose can be carried out using conventional fermentation technology with the yeast, *Saccharomyces cerevisiae*. The theoretical ethanol yield by yeast fermentation is 0.51 g ethanol/g hexose fermented. *S. cerevisiae* cannot ferment D-xylose or arabinose to ethanol, which makes it unsuitable for fermentation of hardwood hemicellulose to ethanol, because of the high xylose content. The enzymatic conversion of D-xylose to xylulose, which can be fermented by *Saccharomyces* spp., has been used with some success, although the D-xylulose fermentation rate is significantly slower than that of hexose fermentation (Hahn-Hägerdal *et al.*, 1986). Ethanolic fermentation by *S. cerevisiae* is also subject to inhibition by the aromatic lignin-degradation products present in steam-pretreated wood hydrolysates (Ando *et al.*, 1986).

Before 1981, there were no known yeasts capable of D-xylose fermentation. *Pachysolen tannophilus* was the first yeast reported to ferment D-xylose to ethanol (Schneider *et al.*, 1981). This initial report started a search to find other yeasts capable of D-xylose fermentation to ethanol with high yields (Maleszka and Schneider, 1982; Suihko and Drazic, 1983; Dellweg *et al.*,1984; duPreez and Prior, 1985). Two other yeasts, *Candida shehatae* and *Pichia stipitis*, were identified as capable of producing high ethanol yields from xylose at rates faster than *P. tannophilus* (duPreez and van der Walt, 1983; duPreez *et al.*, 1984; duPreez *et al.*, 1986; Delgenes *et al.*, 1986; Parekh *et al.* 1986). Both *P. tannophilus* and *C. shehatae* produced significant quantities of xylitol in addition to ethanol, which reduced their final ethanol yields (duPreez and van der Walt, 1983; duPreez *et al.*, 1984).

A review of the literature discussed above indicated that the best known yeast for ethanol production from aspenwood hemicellulose was *Pichia stipitis*. Therefore, in the last year of this project our efforts in hemicellulose fermentation were concentrated on ethanol production from steam-pretreated aspenwood water-solubles (SEA-WS) using this yeast.

Xylose fermentation by *P. stipitis* requires a well defined, although slight, amount of aeration for maximum ethanol production. Insufficient aeration can lead to xylitol formation, while excess aeration can lead to increased yeast cell production or even ethanol consumption. When sufficient aeration was present during xylose fermentation, *P. stipitis* produced little or no xylitol and ethanol yields were reported near the theoretical limit of 0.46 g ethanol/g D-xylose fermented (Dellweg *et al.*, 1984; duPreez and Prior, 1985; Parekh *et al.*, 1986). The required low level of aeration for efficient xylose fermentation by *P. stipitis* is a major process difference from the conventional anaerobic fermentation of hexoses by *S. cerevisiae*.

P. stipitis is capable of fermenting all of the hexoses and cellobiose that may be present in SEA-WS (du Preez *et al.*, 1986). This yeast has been reported to produce xylan degrading enzymes, which would assist the combined hydrolysis and fermentation of xylan in SEA-WS (Lee *et al.*, 1986). *P. stipitis*, like all other known yeasts, cannot ferment arabinose to ethanol. It can, however, use this pentose for cell growth.

In all of the xylose-fermenting yeasts reported to date, xylose fermentation is subject to glucose inhibition and therefore total wood hydrolysate fermentation would not be efficient unless a very long fermentation time is used. The current state-of-the-art in xylose fermentation has recently been reviewed (Skoog and Hahn-Hägerdal, 1988).

Cellulose hydrolysis and fermentation to ethanol

The residue remaining after water and alkali extraction contains 84% cellulose (Forintek Canada Corp., 1984) and is used for both enzyme production and enzymatic hydrolysis to sugar. Hydrolysis of cellulose suffers from two major problems, long residence time and low specific activity of the enzyme.

Hydrolysis of cellulose is complicated because cellulose is a solid whereas the enzymes are soluble. The first step in hydrolysis is adsorption of the enzyme onto cellulose (Lee and Fan, 1982). The accessibility of the cellulose to the large enzyme molecule is therefore an issue in hydrolysis. Cellulose hydrolysis generally proceeds at a rapid initial rate that slows down considerably as hydrolysis proceeds. This change in rate is probably the underlying cause of long residence times and an in-depth investigation of factors governing rate and extent of hydrolysis formed a large part of the project.

Preliminary work with higher initial substrate concentrations has shown that increased amounts of pretreated aspenwood posed considerable problems. The pretreated material has a low bulk density, and as such, caused numerous problems related to material handling. The increased amount of cellulose also required increased concentrations of enzymes and the problems of adsorption of the enzyme and crystallinity of the substrate were more pronounced. To successfully hydrolyse and ferment 15% lignocellulosics may require extensive clean-up of the material since residual inhibitors could pose a problem at these high concentrations. In order to overcome this problem, we envisage the removal of the lignin prior to the CHF process.

Once the cellulose has been hydrolysed, the glucose can be fermented to ethanol using the yeast, *Saccharomyces cerevisiae*. The hydrolysis and fermentation can be performed in the same reactor using a process termed combined hydrolysis and fermentation, CHF, (also termed simultaneous saccharification and fermentation). This technique removes the glucose from the hydrolysis reaction and is beneficial for the rate and extent of hydrolysis (Takagi *et al.*, 1977). The major problem with the CHF technique is the incompatibility of the two reactions. The hydrolysis reaction has a temperature optimum of 45°C whereas the fermentation reaction has a temperature optimum of 30°C.

The enzymatic hydrolysis of cellulose in steam treated aspenwood seldom goes to completion and the waste generated by this stream is substantial. This waste stream is composed of residual cellulose, lignin and yeast and may be ideally suited as a substrate for enzyme production. The feasibility of using this waste stream as a substrate for enzyme production was investigated as it would have a major impact on the cost of the process.

The production of enzymes is reported to be the major cost factor associated with this process, (Nystrom, 1984; Isaacs, 1984). A lot of research has been done on the optimization of cellulase enzyme production from the fungus *Trichoderma reesei*. This fungus was first isolated in 1960 and has undergone an exhaustive mutation program. Fermentation requirements for this organism have also been extensively studied and means have been developed to achieve high cellulase productivity. *T. reesei* suffers from one major disadvantage, low β-glucosidase production, so exogenous β-glucosidase is generally added to the hydrolysis reactor.

A screening program initiated by Forintek in 1980 selected several fungi that produced extracellular cellulase enzymes (Saddler, 1982). The most promising isolate was *T. harzianum* E58 because it produced a complete cellulase complex that was capable of degrading crystalline cellulose to glucose. This fungus produces high levels of extracellular β-glucosidase which is a major advantage over the *T. reesei* fungus. *T. harzianum* also produces high levels of xylanase enzymes required for the degradation of the hemicellulose polymers to monomeric sugars. A major portion of this project determined the feasibility of basing a cellulose conversion process on this organism.

The enzymes are induced by natural substrates and being extracellular in nature, they can be readily harvested, concentrated, and filter-sterilized (if necessary) for use in the hydrolysis process. The ease of production, storage and use of the enzyme preparations also suggests that they are well suited for scale-up studies beyond laboratory investigation. The high efficiencies of enzymatic hydrolysis of both cellulose and hemicellulose substrates also ensure that a single enzyme preparation can be used for hydrolysing the total carbohydrates present in lignocellulosics substrate.

2.2 Background on Trichoderma genus

Trichoderma spp. are widely distributed all over the world and occur in nearly all soils or other natural habitats, especially those containing or consisting of organic material. Qualitative studies on the distribution and occurrence of individual species were hampered by taxonomic uncertainty prior to 1969 and by the lack of precise techniques and culture media for their isolation and enumeration.

Trichoderma viride is the most frequently encountered green species growing on fallen branches and other substrates and has been unanimously regarded as the most typical representative of this genus (Rifai, 1969).

The first report of *T. viride* in soil was made in 1902 and it soon became evident that this genus was the main component of the soil mycoflora. The majority of mycologists adopted the name *Trichoderma viride* to any green spored species of *Trichoderma*. By the late 60's it seemed unjustifiable to over-simplify the taxonomy of *Trichoderma* and to ignore completely the taxonomic value that can be attached to the minor differences shown by these genetically distinct species (Papavizas, 1985).

T. harzianum differs from *T.viride* in many ways, such as absence of spore ornamentation, slightly smaller spore size and paler coloration of their colonies. Older cultures of *T.viride* typically emit a distinctive "coconut" odour of varying strength which makes it comparatively easy to recognize this species (Domsch *et al.*, 1980).

The organism used in many of the cellulose hydrolysis research, first isolated by Reese and Mandels, was originally assigned as *T. viride* but then renamed as *T. reesei*. This organism was deposited at the American Type Culture Collection (ATCC) and is the parent strain for various mutation programmes. Bissett (1983) has reexamined the type strain from ATCC and concluded that this organisms is in actuality *T. longibrachiatum*.

There is no genetic relationship between the mutated *Trichoderma* used in much of the cellulase research field and our strain of *Trichoderma harzianum*. *T. harzianum* is of interest to the agricultural sector because it has potential as a biocontrol agent (Papavizas, 1985). The optimum temperature for growth is 15-35°C, but 30°C represents a good average for most isolates. The maximum growth temperature is 30-36°C and the optimum pH is between 3.7 and 4.7 at normal CO_2 partial pressure. Good growth occurs on mannose, galactose, xylose, sucrose, cellobiose, mannitol, starch and cellulose. Carbon dioxide and ethanol are produced during growth. The organism has antagonistic properties and it was observed that hyphae coil around or invade the hyphae of several tested fungi (Domsch *et al* 1980).

T. harzianum E58 is a wild-type strain and it cannot be directly compared with the mutants of *T. reesei* that are currently used in cellulose hydrolysis. The first step in increasing enzyme productivity of this organism would be to follow the successful protocol used for *T. reesei*. The optimization of basic parameters such as medium composition and fermentation conditions is required for good enzyme production from this fungus. Once the fermentation conditions have been set, increases in productivity and yield through the use of fed-batch substrate addition should be possible. This protocol has been used to increase productivity in the *T. reesei* strains with considerable success. For *T. reesei*, the basic medium composition work was performed by Sternberg and Dorval (1979), Brown and Halsted (1975) and Ryu *et al*. (1979) while fed-batch addition was established by Allen and Andreotti (1982). *T. reesei* has also undergone an exhaustive mutation program (Gallo *et al*., 1978; Montenecourt and Eveleigh, 1978; Schimenti *et al*., 19831 Shoemaker *et al*., 1981) with the isolation of strains that are hyperproducer mutants. None of the reported mutants have increased specific activity but appear to be hypersecretor mutants with enhanced endoplasmic reticulum (Ghosh *et al*., 1984).

At the time that this project was initiated the low levels of β-glucosidase levels produced by *T. reesei* was a major problem to a commercial cellulose conversion process. The high levels of this enzyme produced by *T. harzianum* and the high xylanase levels were qualities that made this organism very attractive for further study. It was for this reason that considerable effort was directed towards the feasibility of using this organism for a commercial process.

2.3 Cellulose conversion

History

Cellulose hydrolysis research began in an attempt to prevent deterioration from occurring. During the 1940's, American troops stationed in the South Pacific were bothered by the rapid deterioration of their cotton goods. The Army established a long range basic research laboratory at Natick to investigate the nature of the rotting, the causal organisms, their mode of action, and a method of control. The laboratory collected deteriorating items and isolated thousands of microorganisms. The cultures were grown on cotton duck and the strips tested for loss of tensile strength.

The strong interest in cellulose hydrolysis waned in post-war years, but due to the concerted efforts of a small group of scientists, led by Mandels and Reese, research continued and much of the ground work was laid.

The major problem in studying enzymatic hydrolysis of cellulose is the complexity of both the substrate and the enzyme system. Cellulose is insoluble and complex containing both amorphous and crystalline regions. The term "crystalline region" refers to areas of tight hydrogen-bonding, where the cellulose chains are arranged in a highly ordered manner. Areas of looser binding are termed "amorphous" or paracrystalline regions.

Enzymatic hydrolysis of crystalline cellulose is biphasic; characterized by an initially rapid rate of reaction, followed by a dramatic reduction in this rate. This decline is a function of both the changing behaviour of the enzyme system towards the substrate and alteration in the substrate.

Wood is mostly crystalline cellulose and is inaccessible to enzymatic attack without some form of pretreatment. The crystallinity of the cellulose and the close association with lignin are the major factors restricting enzymatic hydrolysis. These and other structural features that limit wood susceptibility to enzymatic attack have been outlined by Cowling (1975):

1) Degree of crystallinity of the cellulose
2) Moisture content of the fibres
3) Unit cell dimensions of the crystallites
4) Degree of polymerization
5) Conformation and steric rigidity of the anhydroglucose units
6) Nature, concentration and distribution of substituent groups
7) Nature of associated substances.

Currently, Klesov (1986) includes surface area, while Stone *et al* (1969) and Grethlein (1985) list pore size of the substrate as major factors limiting hydrolysis. Both these factors relate the size and diffusibility of the enzyme molecule to the size and surface properties of the cellulosic material. "Cellulase" itself is a complex of enzymes acting synergistically. The three main cellulolytic components produced by *Trichoderma* spp. are: endoglucanase, exoglucanase and β-glucosidase. A multiplicity of each of these enzymes exists - it is not yet known if the individual forms are genetically determined, or if they arise from differential modification by proteolysis or glycosylation.

Wood (1975) elegantly demonstrated that cellulase act synergistically to hydrolyse fibrous cotton to glucose. When one considers synergism, a description of the substrate must also be included. The structural characteristics of a substrate determines the relative importance of synergism. Degradation of amorphous cellulose can be effected by either exo- or endoglucanase acting independently, whereas crystalline cellulose requires the synergistic interaction of these enzymes (Coughlan, 1985).

The mechanism of synergistic interaction on crystalline cellulose is not fully understood. For efficient hydrolysis of amorphous cellulose the quantity of enzyme is the limiting factor. With crystalline substrates it appears that both the quality of the endoglucanase (discussed below) and the quantity of the exoglucanase components are important.

Klesov *et al* (1986) investigated the adsorption of endoglucanase, produced by different organisms, on a number of pure cellulose substrates varying in their crystallinity and surface area. The endoglucanases fell into two classes those that adsorbed strongly and those that adsorbed weakly. They concluded that the decisive factor in effective hydrolysis of amorphous cellulose is the amount and activity of the enzyme bound to it whereas the hydrolysis of crystalline cellulose depended not only on the extent but also on the quality of binding of the endoglucanase. Strongly adsorbed endoglucanases were found in the cellulase systems that are strongly active against crystalline cellulose, while the weakly adsorbed enzymes were found in systems that were relatively inactive against these substrates.

Wood (1982) studied this phenomenon from a different angle. He examined cross-synergism of cellobiohydrolase and endoglucanse components, from strong and weak cellulose degraders. The source of endoglucanase had a decisive role in determining the extent of hydrolysis: endoglucanase derived from strong degraders strongly solubilized the cotton, while endoglucanase purified from weak cellulose degraders caused little or no solubilization. Apparently, there is a lack of synergy between the cellobiohydrolase and the weak endoglucanase. The major difference between the two types of endoglucanase is that cellobiohydrolase is not produced by the weak degraders.

Both endo- and exoglucanases are strongly inhibited by cellobiose, the product of their concerted action and moderately inhibited by glucose. The sensitivity of the cellulase enzymes to the inhibitors varies drastically with the organism under study. β-Glucosidase is strongly inhibited by its product, glucose.

There are a number of ways to circumvent end-product inhibition such as addition of supplemental β-glucosidase which greatly reduces cellobiose accumulation and enhances glucose yield. Combining hydrolysis with fermentation also removes the inhibitory glucose and the cellulase enzymes are able to operate faster and for longer periods of time.

Enzymatic hydrolysis of cellulose is a long term process and as the reaction time for hydrolysis increases the amount of active enzyme decreases, imposing a restraint on hydrolysis. There is some indication that enzymes are more stable in the presence of substrate, due to adsorption onto the substrate. The extent to which thermal inactivation hinders the enzymatic reaction is mainly dependent on the source of the enzyme system.

Lee and Fan (1982) divided the hydrolytic reaction into the following steps:

1) mass transfer of the enzyme molecule from the bulk aqueous phase to the surface of the cellulose particle

2) enzyme adsorption to the surface of the cellulose and formation of the enzyme-substrate complex
3) transfer of water molecules to the active site of the enzyme-substrate complex
4) surface reaction between water and cellulose promoted by the enzyme-substrate complex
5) transfer of the soluble products, glucose and cellobiose, from the cellulose particles to the bulk aqueous phase
6) decomposition of cellobiose to glucose in the aqueous phase.

During hydrolysis the structural features of the substrate are changing along with the composition and concentration of the adsorbed enzymes. The drastic decrease in initial rate of hydrolysis is attributed to several factors including: the transformation of the substrate into a less digestible form, inhibition of the enzyme action and a decrease in the amount of adsorbed enzyme. As adsorption of the enzyme to the surface of the substrate is a prerequisite for hydrolysis, a decline in the number of binding sites would retard the rate of hydrolysis.

The mechanism of adsorption of the cellulase onto cellulose is not fully understood. Ionic bonding, hydrogen bonding, hydrophobic interactions and permeation effects are all involved in the adsorption of cellulase onto cellulose (Reese, 1982; King, 1965).

As the drop in initial rate of hydrolysis is a severe constraint to this technology a lot of research effort was directed at determining the major factor responsible.

2.4 Techno-economic state of the art - 1984 (all values in US $,1984)

A literature assessment of the techno-economic feasibility of producing ethanol from biomass using similar processes as those under study by Forintek was undertaken at the start of the project. The best available economic assessment of the steam explosion process has been performed by the Solar Energy Research Institute (SERI) (Nystrom, 1984 and Isaacs, 1984) and is based on data provided by Iotech. The basic assumptions of their model were quantitative recovery of cellulose from the gun, an 80% conversion of cellulose to glucose and a 95% conversion of glucose to ethanol. Steam explosion performed by Iotech was done with an acid pre-soak and under their proprietary conditions.

The base case for the economic analysis assumed a 2.0 MBTU/ODT of wood as the steam requirement. The feedstock price was taken to be $30/ODT. The by-product credit for the lignin hemicellulose stream was calculated as fuel (lowest possible case) and gave a credit of 15 cents/gal for the lignin while the furfural was credited at 10 cents/gal. These credits reduced the net production cost to 93 cents/gallon.

The production of enzymes was dependant on a fed-batch addition system that uses high concentrations of biomass. The final cellulose concentration was 150 g/L with a fermentation time of 13 days. The solids generated in this step were sent to the heat generating plant or alternatively a portion could be used for nutrient replacement.

The enzyme production step has a high capital cost due to the use of stainless steel vessels (needed for sterilization purposes) and a 47% reduction in cost could be achieved if carbon steel was used. High enzyme production costs were mostly related to the high costs of the fermentation vessel and agitation and to a lesser extent to the feedstock and nutrient costs. The major areas of concern in the enzyme production step were the physical requirements of the fermentation vessel and more basically the low productivity and specific activity of the enzyme.

The next important parameter studied was the enzyme recycle step. This step was deemed important since without 60% recycle the final ethanol cost would increase by 51 cents/gallon. Enzyme recovery was performed with a counter current continuous flow method which used 25% of the incoming hydrolysis feed as the adsorbent. The analysis assumed 60% recovery in this way but there still exists many unknowns to this hypothesis. The major areas of concern were the stability and half-life of the enzymes, the partitioning of enzymes by this method (β-glucosidase is not adsorbed), the effect of ethanol on the adsorption onto cellulose and the hydrolytic capacity of the recycled enzymes.

The final step was the actual SSF and the analysis of this step assumed an 80% hydrolysis of cellulose to glucose in 48 hours, 90% conversion of glucose to ethanol, solids loaded at 12.5% weight to give an ethanol concentration of 5.5%. Enzyme loading was at a concentration of 25 FPU/g solids with 60% of the enzyme recycled and 40% from fresh sources. The initial yeast concentration was 2 g/L and the yeast was recycled. With these assumptions they calculated a net production cost of $1.30/gallon and a selling price of $1.84 (15% ROI).

The major problem with the assessment was the uncertainty of the base case numbers since little information was available. More information is needed on enzyme loading, residence time, percent conversion of the cellulose to ethanol, stability and recycle of enzymes, accessibility of cellulose if steam explosion was performed without acid.

Report Format

The present report will present the relevant data, in publication format, that was obtained during the course of the project. The focus of the work was on the factors which limited hydrolysis, the production of cellulase enzymes, the recycle of cellulase and unhydrolysed cellulose, the fermentation of the hemicellulose stream and a brief market analysis for ethanol, butanol and butanediol.

III. FACTORS LIMITING HYDROLYSIS

SUBSTRATE EFFECTS LIMITING THE EFFICIENCY OF HYDROLYSIS
J.N. Saddler, C.M. Hogan and M. Mes-Hartree. 1986
Third International Pulp and Paper Conference, Stockholm

SUBSTRATE EFFECTS LIMITING THE EFFICIENCY OF ENZYMATIC HYDROLYSIS OF CELLULOSE

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Introduction

The proposed mechanism of cellulose hydrolysis by the synergistic action of multiple enzymes from the endoglucanase, exoglucanase and β-glucosidase components has previously been extensively reviewed. Apart from the deficiencies that are apparent in the methods used for assaying individual and collective cellulase activities, other factors such as the half life of the enzymes, enzyme regulation, growth conditions and duration of enzyme production, all affect the way in which the hydrolytic potential of the various organisms can be measured. Both the method of pretreatment and the nature of the substrate, have been shown to influence how readily the cellulose and hemicellulose components are hydrolysed.

Concepts in cellulose pretreatment are still being developed, while large scale starch pretreatments are so well established that their importance towards starch conversion is sometimes forgotten by cellulose researchers. It is obvious that all pretreatment methods should increase the accessibility of the cellulose component of the biomass substrate to the cellulase enzymes. Since cellulose is a heterogenous porous substrate its rate of hydrolysis is governed by the number of glycosidic bond sites that are accessible to the rather large cellulase complex. An effective pretreatment increases the number of available sites for cellulase action and promotes the complete hydrolysis of the substrate. It is important not to judge the effectiveness of a pretreatment method by the initial rate of hydrolysis measured as the cellulase hydrolysed in the earlier stages of the reaction may differ substantially in accessibility and composition from the majority of the cellulose.

In our recent work we have tried to establish a continuous cellulose hydrolysis reactor where complete solubilization of the carbohydrate component can be obtained, allowing recycle of the cellulase enzymes for further use. We have found that there is an accumulation of recalcitrant material which cannot be hydrolysed by the addition of fresh enzyme and which selectively adsorbs some of the cellulase components out of solution. The influence of the thermal instability of the enzymes and their adsorption to the recalcitrant residue is compared with some of the structural changes which occur with the substrate.

Results and Discussion

The half life of the cellulase enzymes and the influence of end product inhibition are two of the factors which play a major role in reducing the rate and completion of hydrolysis of lignocellulosic substrates. In our work we have used culture filtrates from the cellulolytic fungi *Trichoderma harzianum* as we had previously found that this organism produced a complete cellulase complex capable of converting the majority of the cellulose component to glucose (Saddler, 1982). Previously we had found (Hogan et al, 1983) that the half life of the different cellulase components was unacceptably short when they were used at the optimum temperature (50°C) for the standard assay methods using Filter paper, carboxymethyl cellulose (CMC) and salicin. When the thermal stability of the various enzyme fractions was assayed at 45°C (figure 1), after 12 hours incubation, the endoglucanase and β-glucosidase activities had decreased by less than 15% while the filter paper activity was lowered by about 20%. These, and previous results (Hogan et al, 1984) indicated that the thermal stability of the enzymes played a relatively minor role in the rate and completeness of hydrolysis, provided the incubation temperature was low enough that significant inactivation of the enzyme did not occur.

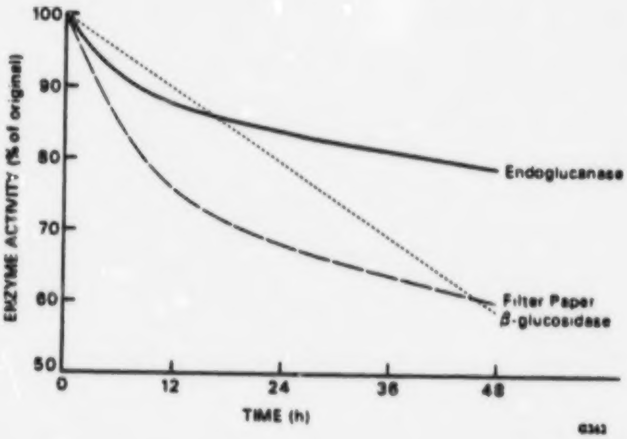

Figure 1. Thermal stability of the endoglucanase, filter paper and β-glucosidase activities of *T. harzianum* E58 culture filtrates incubated at 45°C, pH 4.8

The influence of end product inhibition on the rate and extent of hydrolysis was next studied by supplementing the reaction with 2% glucose (figure 2).

The control consisted of a 5% solka floc solution incubated at 45°C with culture filtrates of *T. harzianum* at a concentration of 20 IU of filter paper activity per gram of cellulose. A combined hydrolysis and fermentation (CHF) approach was also carried out under these conditions to find out how the concomittant conversion of glucose to ethanol alleviated end product inhibition.

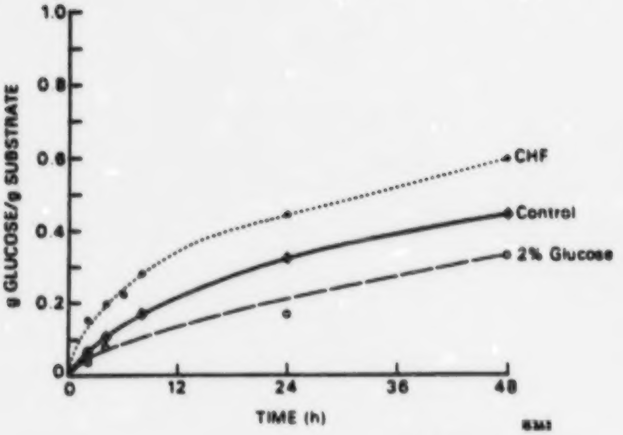

Figure 2. Influence of end product inhibition on the hydrolysis of 5% solka floc BW 300 by culture filtrates of T. *harzianum* E58. The standard hydrolysis had the released sugars removed by a combined hydrolysis and fermentation approach or was supplemented with 2% glucose

Although the initial rate of hydrolysis varied, with the CHF system showing the fastest rate, the rates of hydrolysis in each of the systems after 6-12 hours of incubation were comparable. Longer term incubation indicated that the accumulated glucose affected the extent of hydrolysis as well as the initial rate of hydrolysis. Parallel experiments using cellobiose to supplement the reaction mixtures showed a similar response although it was difficult to determine if in fact the glucose derived from the hydrolysis of the cellobiose caused inhibition as well as the cellobiose itself.

For a soluble substrate, the accessibility of the substrate remains unchanged during the reaction. Therefore, integrated forms of the initial rate expression have been frequently used in predicting the progress of the reaction. For the cellulose-cellulase system however the rate expression obtained from the initial rate studies cannot be applied in predicting the progress of the reaction at the later stage, because the digestibility of cellulose changes drastically as the hydrolysis proceeds due to the changes in the structural features of the cellulose. Decrease in hydrolysis can be attributed to several factors including decrease in the extent of adsorbed enzyme, transformation of the structure of cellulose into a less digestible form as well as the inhibition of the enzyme action by accumulated hydrolysis products.

Lee and Fan (1983) have shown that the initial rate of hydrolysis is directly related to the initial rate of soluble protein adsorption which increases approximately as the specific surface area increases. However, the effectiveness of the adsorbed enzyme to promote complete hydrolysis is directly related to the initial crystallinity index. As there are contradictory reports in the literature with regard to the changes in the crystallinity index (Cr I) of the cellulose as hydrolysis proceeds we examined the CrI of samples taken at different times during the hydrolysis of solka floc. The CrI was measured using x-ray diffraction.

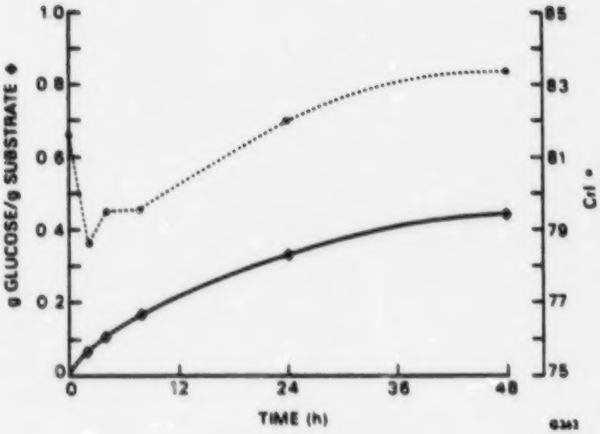

Figure 3. Changes in the crystallinity index (CrI) of solka floc BW 300 during is hydrolysis by culture filtrates of T. *harzianum* E58

The observed decrease in crystallinity which occurred at the initial stages of hydrolysis did not support the theory that cellulase enzymes hydrolyse the amorphous areas of cellulose in preference to the crystalline regions. However the CrI of the residual substrate after 48 hrs hydrolysis was slightly higher than the initial crystallinity of the substrate. If the residue was stored for longer periods of time at 4°C the CrI of the substrate did increase indicating that the adsorbed enzyme preferentially hydrolysed the amorphous component. The reducing sugars detected in solution confirmed that the CrI increased as a result of hydrolysis and not through the "realignment" of the amorphous components.

The first step in the hydrolysis of cellulose is the adsorption of the enzyme onto the substrate. The amount of adsorption is proportional to the extent of hydrolysis or accessibility of the substrate to the enzymes (Lee and Fan, 1983). The ordered structure of the cellulose limits the accessibility of the enzymes to the cleavage sites on the cellulose and the adsorption of cellulases onto the substrate is a measure of the available cleavage sites.

The cellulose samples which were used in the previous studies were also used to determine if there were any changes in accessibility to the enzymes as the hydrolysis proceeded. It was apparent (figure 4) that there was an initial high level of adsorption after which there was a consistent level of adsorption. This trend was similar to the observed changes occurring in the CrI of the substrate. Lee and Fan (1983) have also observed that the intrensic extent of soluble protein remains relatively unchanged throughout the entire hydrolysis period. This indicates that the adsorption sites per unit weight of cellulose does not significantly change during the progress of hydrolysis.

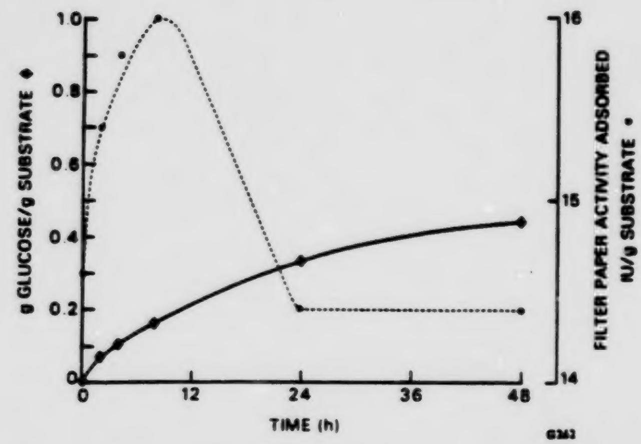

Figure 4. Changes in the adsorption capacity of solka floc BW 300 during its hydrolysis by *T. harzianum* E58 cellulases

It was apparent that as hydrolysis proceeded the residue became more recalcitrant to enzymatic attack. We tried to determine the accessibility of this material by adding fresh enzyme to a 5% concentration of the prehydrolysed substrate (figure 5). We defined accessibility as the yield of glucose that can be obtained from the cellulosic substrate using standard hydrolysis conditions (20 FPU per g substrate, 45°C, 24 hr)

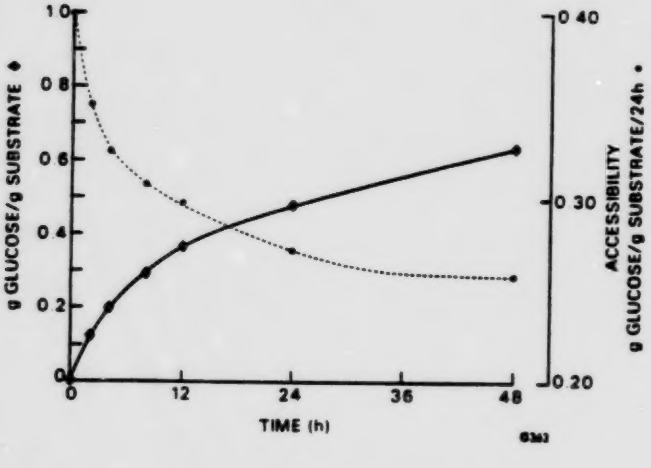

Figure 5. The relationship between the extent of hydrolysis of solka floc BW 300 and the digestibility of the spent cellulose

The results indicated that the accessibility of the cellulose decreased with prolonged incubation resulting in a highly recalcitrant residue. It has been suggested (Lee and Fan, 1983) that the cellulose surface is composed of an active fraction and a less active fraction. Betrabet and Paralikar (1977) have shown that the cellulase enzyme preferentially attacks the 002 and 101 crystal planes. This means that the crystallite width decreases while the crystalline length remained constant during enzyme action. Such a preferential enzyme attack was suggested as being responsible for the market decrease in hydrolysis rate in the latter stages of hydrolysis.

Conclusions

Usually, when native cellulose is degraded by enzyme there is always a residual fraction that survives the attack. This material adsorbs a significant amount of the original enzyme and restricts the reuse of these enzymes on added fresh substrate. It is probable that further mechanical, physical or chemical treatment is required to enhance the rate at which new adsorption sites for the enzyme become available. It is possible that a pretreatment method which not only enhances the initial rate of cellulose hydrolysis but also promotes the complete hydrolysis of the cellulose is required before efficient cellulose hydrolysis can occur.

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USE OF TRICHODERMA HARZIANUM E58 CELLULASES TO DETERMINE OPTIMAL ENZYME CONCENTRATIONS FOR HYDROLYSIS OF SOLKA FLOC BW 300

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USE OF TRICHODERMA HARZIANUM E58 CELLULASES TO DETERMINE OPTIMAL ENZYME CONCENTRATIONS FOR HYDROLYSIS OF SOLKA FLOC BW300

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SUMMARY

Initial enzyme adsorption of Trichoderma harzianum E58 cellulases onto Solka Floc BW300 was examined. The enzyme loading where the substrate became saturated with enzyme appeared to be the most economical enzyme concentration to use for hydrolysis, based on sugars released per unit enzyme. Enzyme saturation was reached at 25 FPU/g substrate on Solka Floc BW300, as determined by studying initial adsorption of the cellulase preparation onto the substrate or by employing a combined hydrolysis and fermentation (CHF) process. The enzymes that adsorb to the substrate in the first hour of hydrolysis have a strong role in governing the overall reaction. T. harzianum E58 has high levels of β-glucosidase and is able to obtain high conversion of Solka Floc BW300 to glucose. End-product inhibition of the cellulase and β-glucosidase can be more effectively reduced by employing a CHF process than by supplemental β-glucosidase.

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INTRODUCTION

Bioconversion of cellulose requires high cellulase enzyme loadings to counteract factors limiting hydrolysis such as end-product inhibition, enzyme inactivation and substrate recalcitrancy. A major limiting factor is the adsorption of the enzyme onto the substrate. As most of the cellulose is inaccessible to the enzyme, efficient hydrolysis is severely limited by the amount of enzyme adsorbed onto the substrate. The major advantages of high enzyme loadings are faster rates of hydrolysis, increased sugar yields and greater resistance to end-product inhibition. The main drawback is the high cost of the enzyme (Wilke et al. 1981). If the minimal amount of enzyme needed to obtain high conversion rates for a given substrate could be determined, the cost of a cellulose-to-glucose process would be significantly reduced.

Cellulose conversion can be regarded as a two-step enzymatic process, with some of the cellulase components adsorbing onto the substrate and degrading it to soluble oligosaccharides and cellobiose. β-Glucosidase, and the cellulase components, hydrolyze the soluble oligosaccharides and cellobiose to glucose. The optimal enzyme requirement for hydrolysis has to be established for each of these stages.

Adsorption of the cellulases onto the substrate is the initial step in hydrolysis. Other researchers have demonstrated a relationship between the amount of cellulase enzyme initially adsorbed to the substrate, and the long-term hydrolysis of the substrate (Peitersen et al. 1977). Lee and co-workers (1982) report that the initial adsorption of cellulases onto cellulose is one of the most important parameters governing the enzymatic rate of hydrolysis. Similarly, Wald and co-workers (1984) found glucose yield reached a maximum when the substrate became saturated with enzyme. As only the adsorbed enzymes are able to hydrolyze the substrate, the unadsorbed enzymes remaining in solution are wasted. One approach to determine the optimal cellulase loading may be to measure the amount of enzyme initially adsorbed to the fresh substrate.

Although enzyme adsorption can influence the overall rate of hydrolysis, end-product inhibition can seriously limit the efficiency of hydrolysis. Both endoglucanase and exoglucanase are strongly inhibited by cellobiose, the product of their concerted action, and moderately inhibited by glucose, while β-glucosidase is strongly inhibited by its product, glucose (Ladisch et al. 1983). These sugars accumulate during hydrolysis and inhibit the reaction rate. The rate of the reaction can be maintained by adding more enzyme as hydrolysis proceeds. Inhibition can be partially overcome by adding supplemental β-glucosidase.

As most Trichoderma spp. are deficient in β-glucosidase, enzyme from another microorganism is routinely added to alleviate inhibition, and increase the glucose yields (Sternberg et al. 1977). Use of commercial enzymes, however, adds to the cost of the process. Trichoderma harzianum E58 produces high levels of extracellular β-glucosidase, reducing the need for supplemental enzyme (Saddler 1982). Although supplemental β-glucosidase will reduce cellobiose inhibition the problem of glucose inhibition of the exo- and endoglucanases is still a concern.

An alternate method which could be used to alleviate end-product inhibition is to continually remove both the glucose and cellobiose throughout hydrolysis by either a combined hydrolysis and fermentation (CHF) (Saddler et al. 1982; Blotkamp et al. 1978) step, ultrafiltration (Frennesson et al. 1985) or use of aqueous two-phase systems (Tjerneld et al. 1985). These methods can also be used to determine the optimal cellulase loading for hydrolysis, as the action of the cellulases on the substrate can be evaluated without interference by the end-products of the reaction.

In this paper we have examined whether an adsorption method and a combined hydrolysis and fermentation process can be used to set the optimal cellulase loading for hydrolysis of the purified cellulose, Solka Floc BW300. This loading was supplemented with a range of β-glucosidase loadings to determine the optimal cellulase: β-glucosidase ratio for hydrolysis. Use of high levels of supplemental β-glucosidase was compared to a CHF process in terms of enzyme demand.

MATERIALS AND METHODS

Cellulase Enzymes

The fungus, Trichoderma harzianum E58 was taken from the Forintek Culture Collection. A spore inoculum was used to initiate growth in 300 mL shake flasks containing 100 mL Vogel's medium (Montenecourt and Eveleigh 1977) with glucose as the carbon source.
After 2 days incubation, the mycelium inoculum was added to a 30-L New Brunswick Microferm Fermentor, containing Vogel's medium with 1% Solka Floc BW300 as the carbon source. The fungal cultures were harvested when peak cellulase activity was reached, and filtered through a Whatman 934-AH glass fiber filter. The cellulase was concentrated by ultrafiltration using a Pellicon Cassette System (Millipore) fitted with a polysulfone membrane with a molecular weight cut-off of 10,000 daltons. The retentate was freeze-dried at -70°C and stored at -10°C.

The purified exoglucanases and β-glucosidase were kindly donated by Dr. L.U.L. Tan. They were purified from culture filtrate of T. harzianum E58 following the methods outlined by Tan and co-workers (Tan et al. 1986; Tan et al. 1987).

Assays

Total reducing sugars were determined colorimetrically using dinitrosalicylic acid reagent (Miller 1959). Glucose was determined colorimetrically by the Glucostat enzyme assay (Raabo and Terkildsen 1960). Soluble protein was determined after TCA precipitation by the method of Lowry et al. (1951) as modified by Tan et al. (1984) using bovine serum albumin (Sigma) as standard. Ethanol was determined on a gas-liquid chromatograph (Varian 4600) fitted with a 6 ft x 1/8 in Chromosorb 101 column (Chromatographic Specialities) and a flame ionization detector. The detector was kept at 250°C, the column oven at 180°C and the injector port at 200°C.

Helium, saturated with formic acid (Ackman 1972), was used as the carrier gas at a flow rate of 30 mL/min.

The different cellulase activities were measured using the modified method of Mandels (1976) adapted by Saddler et al. (1985). Endoglucanase (1,4-β-D-glucan-4-glucanohydrolase, EC 3.2.1.4) and β-glucosidase (EC 3.2.1.21) activities were assayed using final concentrations of 0.5% (w/v) carboxymethylcellulose (medium viscosity, Sigma Chemical Co.) and salicin (Sigma Chemical Co.), respectively. The hydrolyses were carried out at 50°C for 30 min in 50 mM sodium citrate buffer, pH 4.8.

Filter paper activities were measured by adding 1 mL culture supernate to 1 mL 50 mM sodium citrate buffer, pH 4.8, containing a 1 cm x 6 cm strip (50 mg) Whatman No. 1 filter paper. The hydrolyses were carried out for 1 h at 50°C.

The enzyme assays were terminated by addition of dinitrosalicylic acid reagent. Activity units were expressed as micromoles of glucose equivalents produced per minute.

Saccharification Studies

Solka Floc BW300 (Brown & Co., Berlin, NH) and buffer were added to the reaction flasks and autoclaved 15 min at 121°C. The enzyme solutions were filter-sterilized through a 0.45 μm nylon membrane (Nalgene) then aseptically added to the reaction flasks. Hydrolysis was routinely carried out at 45°C, with an agitation speed of 140 rpm. The systems were maintained at pH 4.8 using 50 mM sodium acetate buffer. The enzymatic reactions were terminated by boiling the systems for 10 min.

Combined Hydrolysis and Fermentation Process

Saccharomyces cerevisiae C495 (NRCC 202001) was grown aerobically at 28°C, shaken at 110 rpm, using the medium of Rainbow (1970). Wheaton serum vials containing 2% Solka Floc BW300 and 0.2% yeast extract (Difco) in 50 mM sodium acetate buffer, pH 4.8, were flushed with N_2, sealed with butyl stoppers and aluminum caps, and autoclaved at 121°C for 15 min. The cellulases, dissolved in 50 mM sodium acetate buffer, were filter sterilized, and aseptically injected into the vials. A 5% inoculum of 2 day old S. cerevisiae culture was added immediately. The vials were incubated at 37°C, 140 rpm for four days. The contents of the flasks were assayed for reducing sugars, glucose and ethanol. The percent conversion based on the ethanol yield was calculated as follows:

$$\%\ \text{Conversion} = \frac{\dfrac{\text{EtOH (mg/mL)}}{46.07} \times \dfrac{180}{2} \times \dfrac{1}{\text{Biomass Factor*}}}{\text{Conc. of initial substrate} \times \dfrac{180}{162}}$$

Adsorption Studies

The designated quantity of Solka Floc BW300 was suspended in 50 mM sodium acetate buffer, pH 4.8, and left to stand overnight at 28°C. The cellulase enzyme solution, at 28°C, was added to the substrate. Adsorption studies were routinely carried out at 28°C, 150 rpm for 10 min, after which time the system was vacuum filtered for 15 s through Whatman 934 AH glass microfiber filters, to remove the cellulose residue. Controls for these experiments consisted of enzyme incubated without cellulose for 10 min under the same incubation conditions. Adsorbed protein and enzyme were calculated by subtracting the recovered protein and enzyme in solution from the control.

RESULTS AND DISCUSSION

Enzyme Loading

A hydrolysis curve for T. harzianum E58 cellulases on Solka Floc BW300 at various enzyme loadings was established (Figure 1). At low enzyme loadings the amount of hydrolysis obtained per filter paper unit (FPU) was high. At enzyme loadings over 50 FPU/g little additional hydrolysis was achieved with additional enzyme. The fact that enzyme concentrations over 50 FPU did not enhance hydrolysis, even though there was sufficient substrate left for the enzyme to attack, is probably a result of a limitation in the number of binding sites available to the enzyme at a given time. Once these sites are filled the unadsorbed enzymes remain in solution until an accessible site is available. The most efficient amount of enzyme to add for hydrolysis would be reached when all the sites accessible to enzymatic attack are filled with enzyme. The above data suggested that the substrate may become saturated with enzymes at an enzyme loading between 20-50 FPU/g. Therefore, the maximum amount of enzyme that should be used for hydrolysis of this substrate appeared to lie roughly in the range of 20-50 FPU/g.

Adsorption and Hydrolysis

Enzyme adsorption was investigated as a means of determining the optimal enzyme loading for hydrolysis. The critical problem in relating adsorption to hydrolysis is the difference in the nature of the two processes. In adsorption one captures the substrate at a given moment and examines it; while in hydrolysis one is looking at a continual process. To reconcile the two processes initial adsorption will be examined, as the first step in hydrolysis is adsorption of the enzyme to the substrate.

The capacity of Solka Floc BW300 to adsorb T. harzianum E58 cellulases was determined, and compared with its hydrolysis after 8 hours, over a range of enzyme loadings (Figure 2). The amount of endoglucanase and filter paper activities that was adsorbed increased with added enzyme up to a loading of 25 FPU/g. At higher concentrations, the amount of adsorbed endoglucanase and filter paper activities levelled off. A similar profile for hydrolysis of the substrate to reducing sugars was obtained, with an increase in the sugar concentrations up to a loading of 25 FPU. At higher enzyme concentrations, the amount of sugars released levelled off. The enzyme loading at which saturation of the substrate occurs appears to correlate with the optimum loading for hydrolysis. Additional enzyme did not increase the yield, so it appears that the adsorptive process is controlling the overall rate of hydrolysis.

Substrate Saturation

A potential problem in using adsorbed enzymes to determine optimal enzyme loading for hydrolysis is the heterogeneous nature of the cellulase system. The various components of the cellulase complex are adsorbed in different proportions to the substrate, and have different binding affinities (Kyriacou et al. 1988). For hydrolysis to be feasible, however, all the components of the cellulase system must be present.

When Tan and co-workers investigated the enzyme system of T. harzianum E58 they found that the major cellulase enzyme secreted by this fungus was an exoglucanase. In addition, this enzyme has a stronger affinity for the Solka Floc BW300 than did the endoglucanases. Using culture filtrate the majority of the adsorbed proteins were found to be the exoglucanases.

In our work, the purified exoglucanase, derived from T. harzianum E58, was used as a binding probe to determine the substrate saturation level. Varying concentrations of purified exoglucanase were added to Solka Floc BW300, and the amount of adsorbed protein determined (Figure 3). Adsorption increased with added protein up to 15 mg adsorbed protein/g substrate, after which little additional protein was adsorbed. The substrate was saturated with enzyme at this point, any excess enzyme added either remained in solution or was only loosely associated with the substrate.

The adsorption behaviour obeys the Langmuir Adsorption Isotherm (Gilbert and Tsao 1985), with the amount of enzyme adsorbed increasing with the amount of enzyme added. As a homogeneous macromolecule and purified cellulose were used, the Langmuir Adsorption Isotherm Equation could be applied to predict the maximum amount of exoglucanase able to adsorb to the substrate. This was calculated as 14.4 mg protein/g substrate.

Almost all of the added enzyme adsorbed to the substrate, up to a concentration of 10 mg protein added/g cellulose. At higher enzyme concentrations, a portion of the added enzyme did not bind to the substrate, but remained in solution. At 10 mg protein the majority of the substrate may be covered with enzyme so that additional enzyme may not be able to attach as quickly, or as strongly, due to steric interference by the adsorbed enzymes as well as a reduction in the number of binding sites available. Although the incubation time for adsorption has to be low to avoid hydrolysis of the substrate, it may be insufficient to allow complete adsorption at the higher enzyme loadings. Consequently, the amount of enzyme adsorbed at substrate saturation is less than the amount of enzyme added to obtain substrate saturation. In this case a saturation value of 14.4 mg/g substrate corresponded to an enzyme loading of 35 mg protein/g Solka Floc BW300. In reality, the enzyme loading required for saturation may be lower than estimated by this method.

The relationship between the adsorption of purified exoglucanase and the adsorption of filter paper activity, present in the culture filtrate, was examined next. A direct correlation between protein adsorbed in the culture filtrate could not be made, as other non-cellulolytic proteins are present which would interfere with this assessment. In Figure 2 varying concentrations of culture filtrate were added to the Solka Flow BW300, and the amounts of adsorbed and unadsorbed filter paper activity determined. These values were plotted using the Langmuir Isotherm Equation, and it was determined that the maximum amount of filter paper activity able to be adsorbed was 12.5 FPU/g substrate. This value corresponds to 15.1 mg adsorbed protein/g, which is comparable to that obtained with the purified exoglucanase (14.4 mg/g substrate). These results suggest that the maximum amount of enzyme able to adsorb to the substrate could be determined by examining filter paper activity adsorbed from the culture filtrate.

To determine the minimum amount of enzyme needed for efficient hydrolysis, the saturation level of the adsorbed protein (15.1 mg/g substrate) must be correlated with the total amount of enzyme added. The amount of enzyme which bound at saturation corresponded to an enzyme loading of 23 FPU/g cellulose. As discussed above, the actual enzyme loading to achieve saturation may be lower.

Role of Initially Adsorbed Cellulases in Hydrolysis

We next tried to determine if the initial enzyme adsorption could be used to determine the minimum amount of enzyme needed for hydrolysis. To do so, it was necessary to establish that the initially adsorbed enzymes had a major role in the overall hydrolytic process, and were not just representative of the initial phase. Previously, Lee and Fan (1983) had reported that similar reducing sugar yields could be obtained when the substrate was incubated either with the same enzymes for the entire process, or if the liquid phase, containing the unadsorbed enzymes, was removed after one hour and replaced with buffer. In removing the liquid phase, the β-glucosidase was removed along with the unadsorbed cellulases. Consequently, the ability of the bound cellulases to promote hydrolysis would be severely handicapped by cellobiose and glucose inhibition. To measure the full extent to which the initially bound cellulases control hydrolysis it would be desirable to reduce end-product inhibition by supplementing the system with β-glucosidase. For comparative purposes, a β-glucosidase loading equal to the control should be added.

T. harzianum E58 cellulases were incubated at 4°C for one hour with Solka Floc BW300 to allow maximum enzyme adsorption. An enzyme loading of 26 FPU/g cellulose was used, as this corresponded to the maximum protein adsorption level for the substrate as determined above. After removal of the liquid phase the substrate was washed to remove unadsorbed proteins, and the insoluble residue was lyophilized. Part of the enzyme-substrate complex was resuspended in buffer; the other part was resuspended in buffer and supplemented with purified β-glucosidase at levels equivalent to the original mixture. A control containing the original enzyme activity and substrate was also prepared.

The reducing sugars and glucose released by the adsorbed cellulases supplemented with β-glucosidase corresponded to approximately 90% of the values obtained with the control (Figure 4). The reducing sugars and glucose yields obtained by the adsorbed cellulases, without supplemental β-glucosidase, were only 50 and 25%, respectively of the control. End-product inhibition is responsible for the low sugar yields in the latter system.

It was interesting to note that the cellulases initially adsorbed to the substrate were able to hydrolyze the substrate to an extent nearly equivalent to the control. The enzymes that did not bind, apparently have only a minor role in the hydrolysis of the substrate. This established that the enzymes initially adsorbed to the substrate were primarily responsible for its hydrolysis. These results indicated that the minimal enzyme requirements for effective hydrolysis of Solka Floc BW300 by *T. harzianum* E58 cellulases could be determined by examining the initial adsorption onto this substrate.

End-Product Inhibition

Using the previously determined "optimal" filter paper loading of 25 FPU/g substrate, the effect of supplemental β-glucosidase on hydrolysis was studied. Various concentrations of the purified β-glucosidase were combined with *T. harzianum* E58 cellulases and incubated with Solka Floc BW300 for a 4 day period (Figure 5). Hydrolysis to glucose was enhanced by about 10%, after 24 hours, by the addition of 4 IU β-glucosidase/g substrate.. Additional β-glucosidase had little effect on hydrolysis. The beneficial effect of supplemental β-glucosidase disappeared after 48 hours incubation. The enzyme activity of the control (no supplemental β-glucosidase) was 25.7 FPU and 16.5 IU β-glucosidase/g substrate. For this enzyme system, acting on 2% Solka Floc a filter paper activity to β-glucosidase ratio of 1.6 FPU: 1.0 IU β-glucosidase is sufficient to cope with end-product inhibition.

β-Glucosidase, in converting the cellobiose to glucose, removes the more potent inhibitor of the exo- and endoglucanases, however, glucose levels increase as hydrolysis proceeds. Although glucose only weakly inhibits the exo- and endoglucanase, it must be considered when examining enzyme loading.

Combined Hydrolysis and Fermentation

To avoid the problem of glucose or cellobiose inhibition, during examination of hydrolysis, a combined hydrolysis and fermentation (CHF) process was used. Glucose was continuously removed from the reaction mixture by fermentation to ethanol, greatly stimulating the hydrolysis of the cellulose. Since end-product inhibition was virtually eliminated, the unhindered action of the endoglucanase and exoglucanase on the substrate could be assessed.

Enzyme loadings ranging from 0-30 FPU/g cellulose were examined (Figure 6). The filter paper to β-glucosidase ratio of the cellulase preparation was 1.0 FPU: 1.0 IU β-glucosidase. In one system the yeast, Saccharomyces cerevisiae, was added concurrently with the cellulases, and the fermentation vials incubated at 37°C. A hydrolysis-only system was run in parallel at 37°C to observe the extent to which glucose inhibition limited the reaction.

In the hydrolysis-only system, the amount of sugar released increased with the enzyme loading for the entire range studied. The amount of sugars released per enzyme unit was greater in the CHF systems, especially at the lower enzyme loadings. As the enzyme loading increased above 15 FPU the differences in the sugar yield between the CHF and hydrolysis-only systems narrowed. Twenty to twenty-five FPU has been established as the minimal value to fulfil the endoglucanase and exoglucanase requirements for efficient hydrolysis, so hydrolysis is not limited by insufficient cellulase. In the hydrolysis-only system there was insufficient β-glucosidase at an enzyme loading of 20-30 FPU, therefore, enhanced hydrolysis at the higher enzyme loadings was mainly due to the removal of cellobiose inhibition by the additional β-glucosidase. Since, hydrolysis was run at 37°C, rather than 45°C, the activity of the β-glucosidase was lower and more enzyme was required to avoid end-product inhibition in this experiment than was estimated in the above section.

In the CHF systems the amount of hydrolysis reached a plateau at an enzyme loading of 15-20 FPU/g substrate at both day 2 and 4. Additional enzyme had no effect on hydrolysis, indicating that for efficient hydrolysis of this substrate the maximum amount of enzyme that should be used is 15-20 FPU/g substrate. The enzyme loading corresponding to the plateau point in the CHF process provided an alternate method to initial adsorption to determine the most efficient enzyme loading for hydrolysis of the cellulose.

Supplemental β-Glucosidase

As an alternative to the CHF, the use of supplemental β-glucosidase (at 30 IU β-glucosidase/g Solka Floc BW300) in establishing the optimal enzyme loading was tested. Purified β-glucosidase from T. harzianum E58 was combined with T. harzianum E58 cellulases, ranging from 2.5-50 FPU/g. A control, with no supplemental β-glucosidase, was run in parallel. The supplemental enzyme enhanced the hydrolysis quite dramatically at the lower enzyme loadings, in comparison to the control. The glucose yield for the supplemented and unsupplemented systems coincided at 30 FPU/g, and had equivalent glucose yields at the higher enzyme loadings. The conversion to glucose in the supplemented system did start to level off at the designated optimal cellulase loading of 20 FPU, but there was a steady accumulation of sugars up to about 35 FPU/g substrate.

High levels of supplemental β-glucosidase may have alleviated end-product inhibition of β-glucosidase by glucose, but can not remove the inhibition it exerts on the endo- and exoglucanases. As seen above, the cellulase loading for efficient hydrolysis is reasonably low (25 FPU/g). End-product inhibition, however, is a serious liability to fast rates of hydrolysis, increasing the demand for both β-glucosidase and the cellulases. Removal of the glucose by a CHF process is a more economical way of dealing with end-product inhibition than the use of supplemental β-glucosidase as additional enzyme is not required to meet the demands imposed by end-product inhibition.

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Figure 1.

Effect of enzyme loading on the hydrolysis of a 2% (w/v) concentration of Solka Floc BW300 to reducing sugars (●) and glucose (■) after 4 days incubation at 45°C.

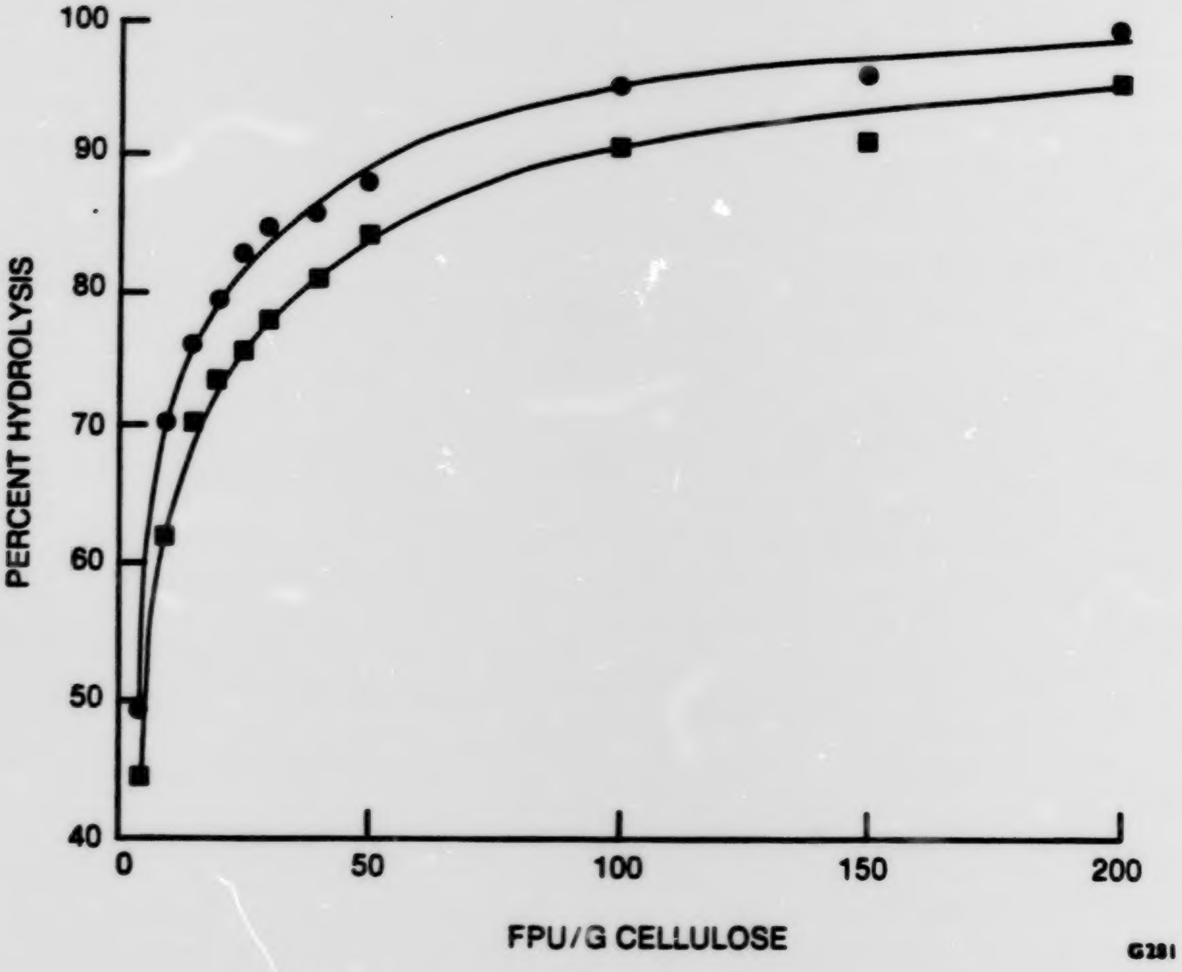
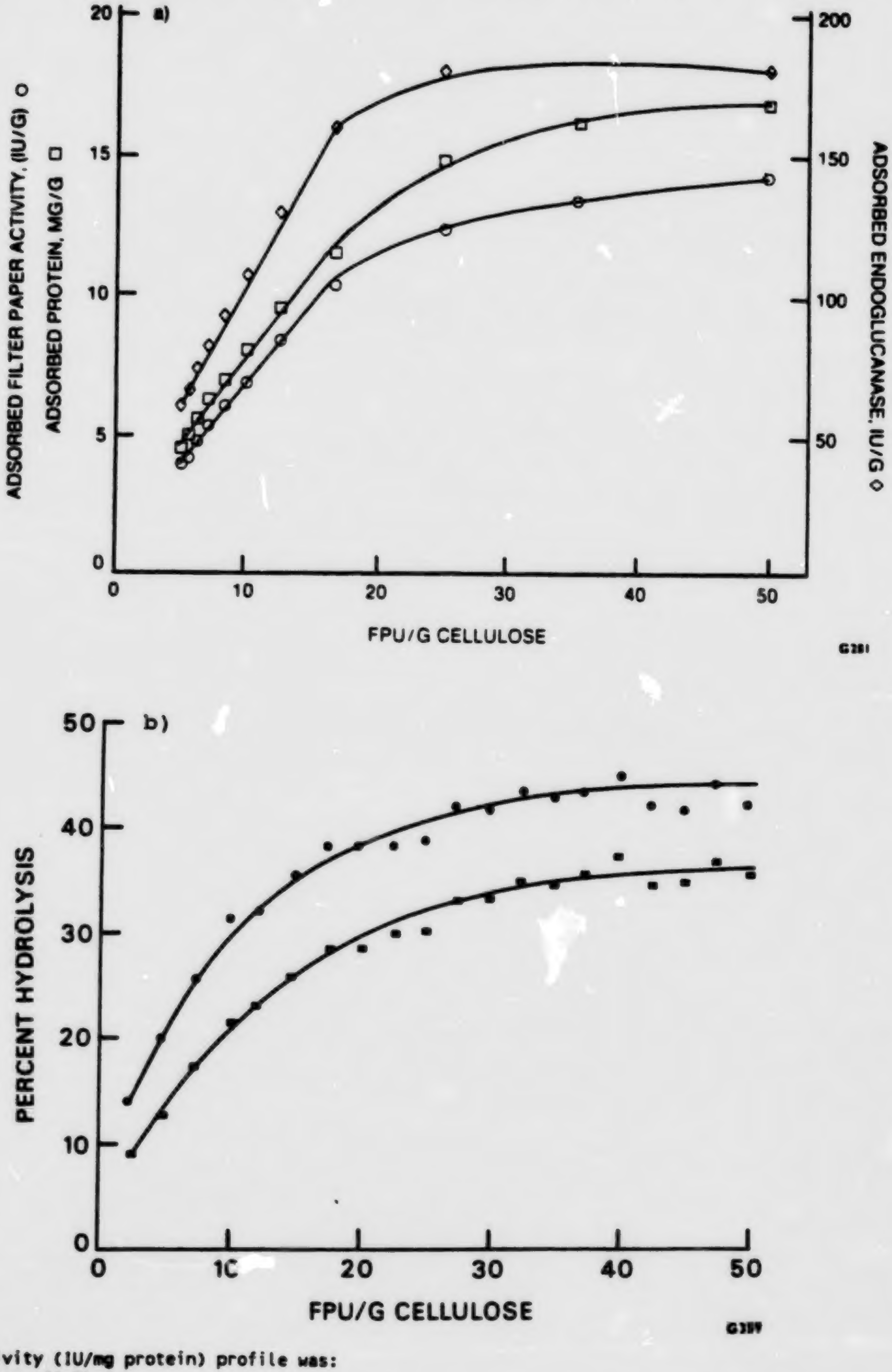


Figure 2.

Relation between a) the amount of enzyme adsorbed onto 5% (w/v) Solka Floc BW300 and b) its hydrolysis to reducing sugars (●) and glucose (■).

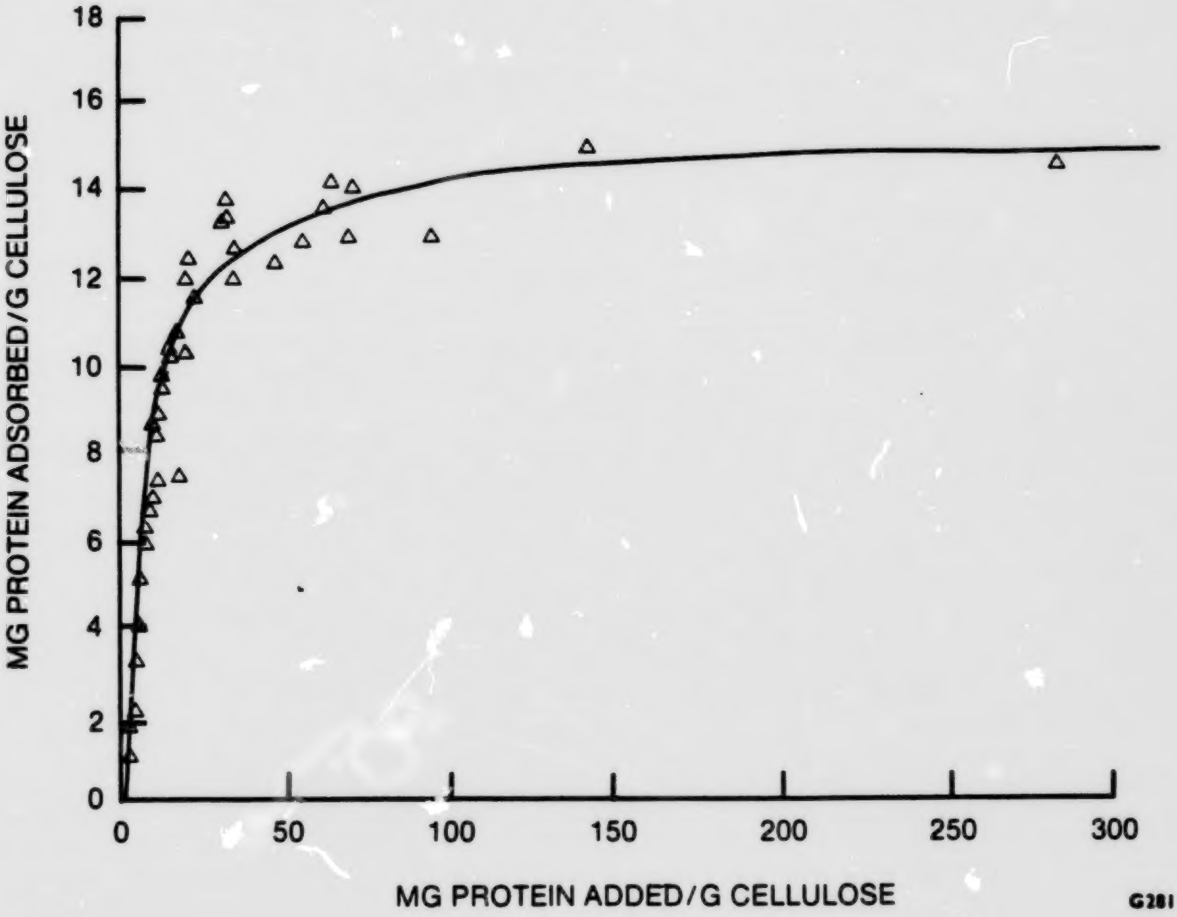


Enzyme activity (IU/mg protein) profile was:
Endoglucanase, 14.51
β-glucosidase, 0.55
Filter paper activity, 0.68

Incubation time for the adsorption study was 10 minutes.
Incubation time for hydrolysis was 8 h.

Figure 3.

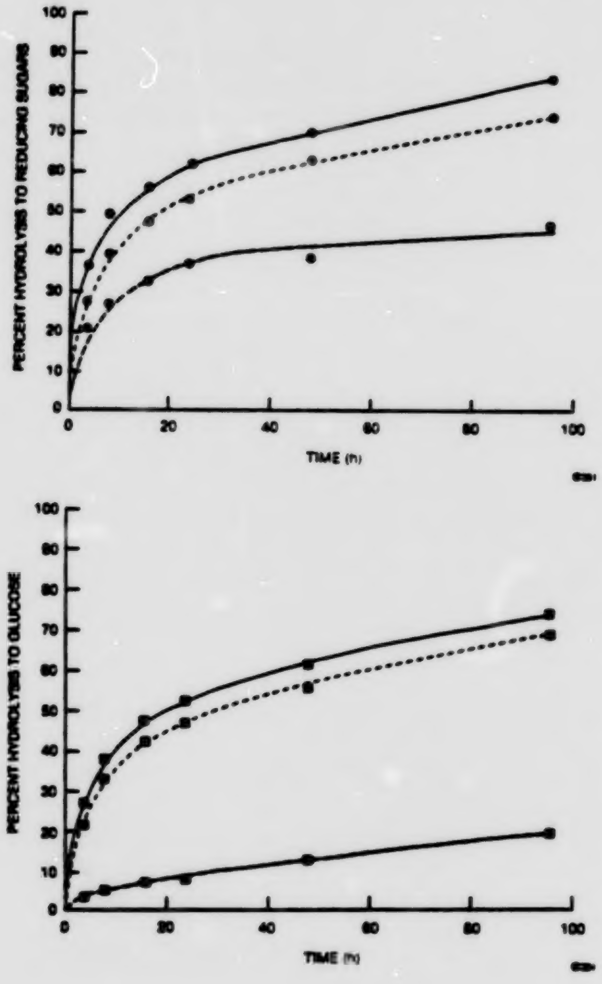
Adsorption of varying loadings of purified exoglucanase, derived from *T. harzianum* E58, onto Solka Floc BW300.



Incubation time for the adsorption study was 10 minutes.

Figure 4.

Hydrolysis of Solka Floc BW300 by adsorbed cellulases after removal of the unadsorbed protein remaining in solution: control (●—●); supernatant removed and replaced with buffer (●- - -●); supernatant removed, replaced with buffer and supplemented with purified β-glucosidase (●—●).

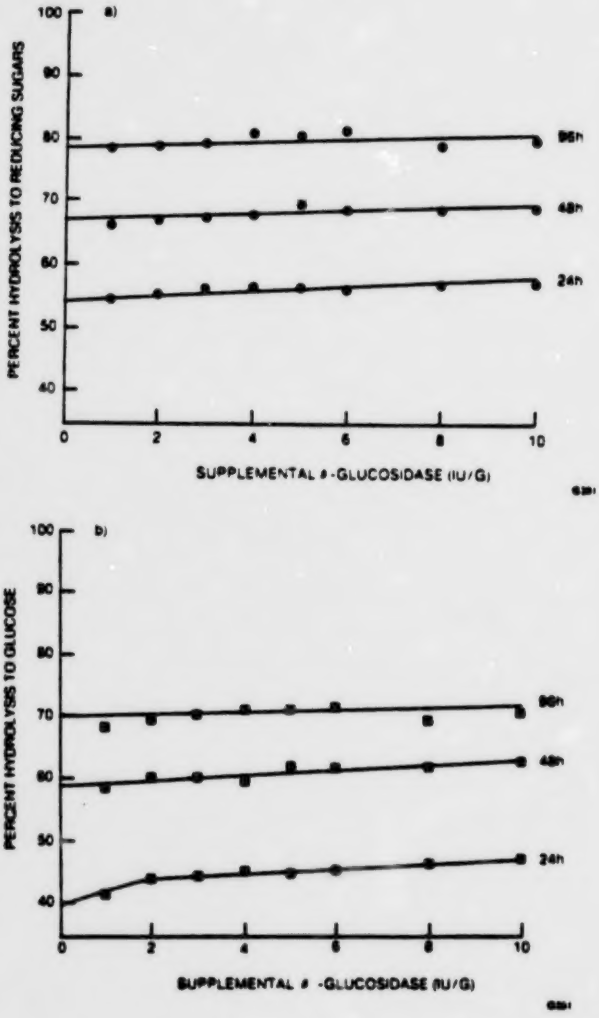


Enzyme loading of 25.6 FPU/g substrate was used.

Enzyme activity (IU/mg protein) profile was:
Endoglucanase, 15.60
β-Glucosidase, 0.56
Filter paper activity, 0.79

Figure 5.

Effect of supplemental β-glucosidase purified from T. harzianum E58 on the long-term hydrolysis of a 2% (w/v) concentration of Solka Floc BW300 to a) reducing sugars and b) glucose.

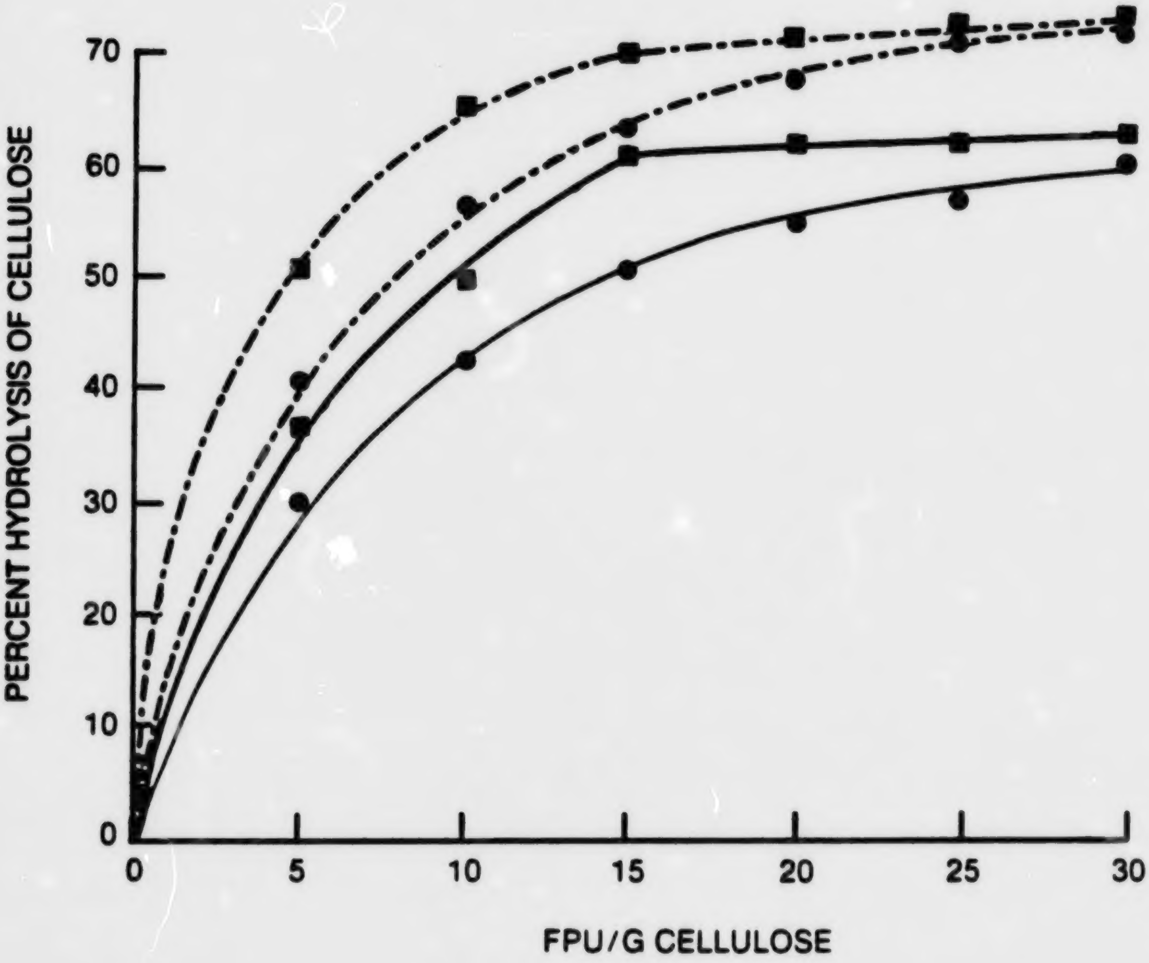


Enzyme activity (IU/mg protein) profile was:
Endoglucanase, 19.07
β-Glucosidase, 0.55
Filter paper activity, 0.85

Enzyme loading was 25.7 FPU/g substrate

Figure 6.

Comparison of a combined hydrolysis and fermentation process (■) and hydrolysis process (●) on the degradation of a 5% (w/v) concentration of Solka Floc BW300 at day 2 (——) and day 4 (-·-) using a range of enzyme loadings.

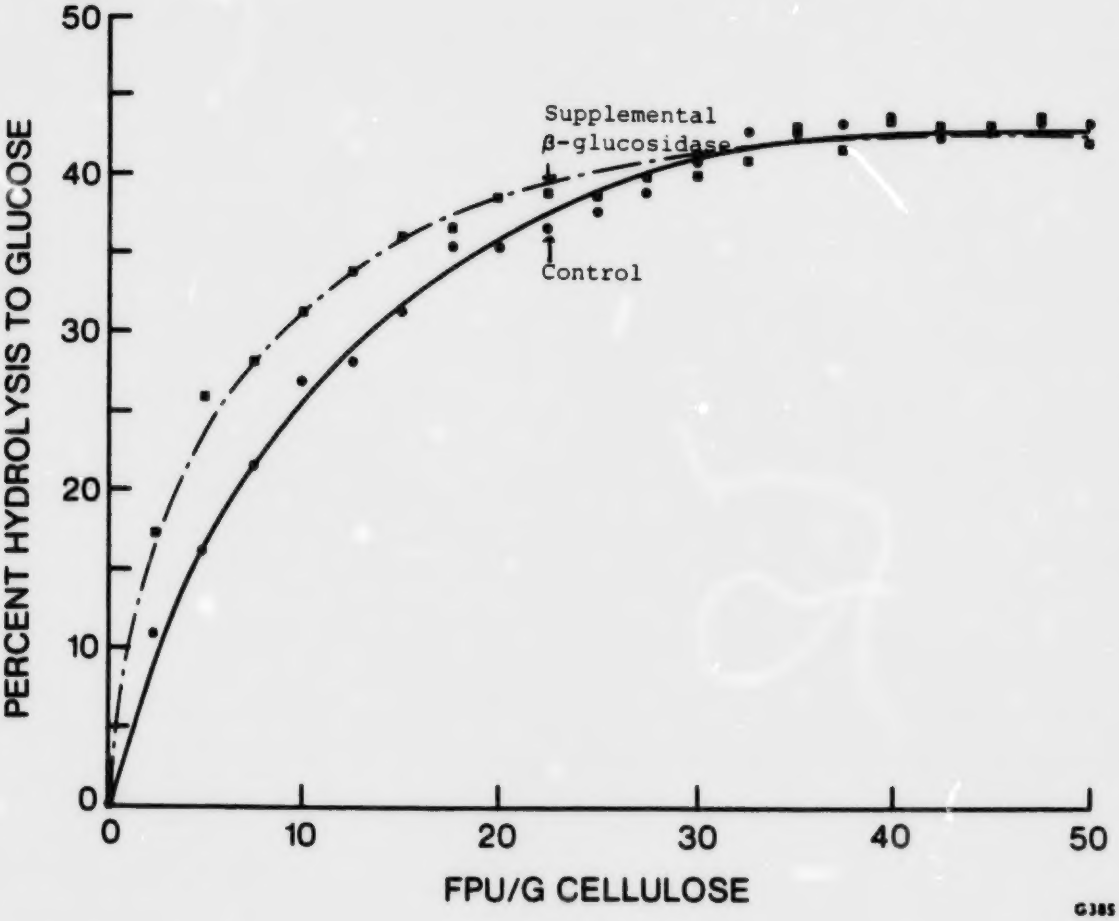


In the CHF systems percent hydrolysis was based on the ethanol yield; no glucose was detected on either day 2 or day 4.

Enzyme activity (IU/mg protein) profile was:
Endoglucanase, 19.87
β-Glucosidase, 0.84
Filter paper activity, 0.87

Figure 7.

Effect of supplemental β-glucosidase on hydrolysis of a 5% (w/v) concentration of Solka Floc BW300 after 2 days using a range of enzyme loadings.



β-Glucosidase loading of 30.0 IU β-glucosidase/g substrate was used.

Enzyme activity (IU/mg protein) profile was:
Endoglucanase, 15.60
β-Glucosidase, 0.56
Filter paper activity, 0.79

IV. RECYCLE- ENZYMES AND CELLULOSE

RECYCLE OF ENZYMES AND SUBSTRATE FOLLOWING ENZYMATIC HYDROLYSIS OF STEAM-PRETREATED ASPENWOOD

M. Mes-Hartree, C.M. Hogan and J.N. Saddler, 1986

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Recycle of Enzymes and Substrate Following Enzymatic Hydrolysis of Steam-Pretreated Aspenwood

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The commercial production of chemicals and fuels from lignocellulosic residues by enzymatic means still requires considerable research on both the technical and economic aspects. Two technical problems that have been identified as requiring further research are the recycle of the enzymes used in hydrolysis and the reuse of the recalcitrant cellulose remaining after incomplete hydrolysis. Enzyme recycle is required to lower the cost of the enzymes, while the reuse of the spent cellulose will lower the feedstock cost. The conversion process studied was a combined enzymatic hydrolysis and fermentation (CHF) procedure that utilized the cellulolytic enzymes derived from the fungus *Trichoderma harzianum* E58 and the yeast *Saccharomyces cerevisiae.* The rate and extent of hydrolysis and ethanol production was monitored as was the activity and hydrolytic potential of the enzymes remaining in the filtrate after the hydrolysis period. When a commercial cellulose was used as the substrate for a routine 2-day CHF process, 60% of the original filter paper activity could be recovered. When steam-treated, water-extracted aspenwood was used as the substrate, only 13% of the original filter paper activity was detected after a similar procedure. The combination of 60% spent enzymes with 40% fresh enzymes resulted in the production of 30% less reducing sugars than the original enzyme mixture. Since 100% hydrolysis of the cellulose portion is seldom accomplished in an enzymatic hydrolysis process, the residual cellulose was used as a substrate for the growth of *T. harzianum* E58 and production of cellulolytic enzymes. The residue remaining after the CHF process was used as a substrate for the production of the cellulolytic enzymes. The production of enzymes from the residue of the Solka Floc hydrolysis was greater than the production of enzymes from the original Solka Floc.

INTRODUCTION

The bioconversion of waste lignocellulosics to higher value products involves the enzymatic hydrolysis of the cellulosic component. One of the major expenses associated with this process is the high cost of the enzymes. Wilke[1] has reported that enzyme production accounts for as much as 60% of the total cost of the process, which makes this part of the process a prime target for research. One avenue that has been suggested as a means of lowering the cost of the enzyme component of the process is the recovery and recycle of the enzymes. Isaacs[2] has indicated that recycle of 60% of the cellulase enzymes could have a major impact on the cost of the enzymes. Little research has been done on the technical problems and feasibility of these levels of enzyme recycle in a process that uses pretreated wood as its primary feedstock. This was the major objective of the present report.

MATERIALS AND METHODS

Microorganisms

The fungus *Trichoderma harzianum* E58 was taken from the Forintek Culture Collection. *Saccharomyces cerevisiae* C495 (NRCC 202001) was grown under standard conditions.

Cellulase Preparation

A spore inoculum was used to initiate growth in shake flasks containing Vogel's medium and 2% glucose. After 2 days at 28°C the mycelial suspension was used to initiate growth in shake flasks or in a 30-L fermentor containing 1% cellulosic material. The incubation period was for 3–4 days at 28°C. The cultures were then filtered through a Whatman GF/F glass microfiber filter. The culture filtrate was assayed for enzymatic activity and used directly for enzymatic hydrolysis experiments. Some hydrolysis experiments were performed with culture filtrates that had been concentrated by the Pellicon filtration system using a membrane with a molecular cutoff of 10,000 daltons and freeze dried.

Assays

Soluble protein was determined after TCA precipitation by the method of Lowry et al.[3] using bovine

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serum albumin as the standard. Total reducing sugars were estimated using the dinitrosalicylic acid assay.[4] Glucose was determined by the glucostat enzyme assay.[5] Ethanol was determined by gas–liquid chromatography using a Chromosorb 101 column with flame ionization. Helium saturated with formic acid was used as the carrier gas.[6] Total nitrogen was measured via the Kjeldahl.

Hexosan was determined by an adaptation of the anthrone method of Koehler[7] in which the time was extended to 15 min to permit the more labile pentosan color to fade. A correction for the residual pentosan color was made.

Enzymatic activity was determined by the method of Mandels et al.[8] One milliliter of a properly diluted culture filtrate was added to 1 mL 0.05*M* citrate buffer at pH 4.8. For the filter paper assay the substrate was a 1 × 6-cm strip of Whatman No. 1 filter paper, while for the endoglucanase assay the substrate was 10 mg carboxymethyl cellulose (CMC medium viscosity, Sigma C-4888) and for the β-glucosidase assay the substrate was 10 mg salicin. All three assays were performed at 50°C. The filter paper assay was incubated for 1 h while the endoglucanase and β-glucosidase assays were incubated for 30 min. For all three assays the reaction was terminated by adding 3 mL dinitrosalicylic acid reagent to each tube and boiling the tubes for 5 min. The tubes were cooled to room temperature and the absorbance read at 575 nm. One unit of enzyme activity was defined as 1 μmol glucose equivalents released per minute.

Combined Hydrolysis and Fermentation (CHF) Procedure

A 5% substrate concentration (based on dry weight) and 0.5% yeast extract were added to a 60-mL serum vial or to a 2-L fermentor. The cellulase enzyme preparation was added at a concentration of 15 IU filter paper activity per gram of substrate. A 3-day-old suspension of *S. cerevisiae* was added at a 10% inoculum level. The fermentor and vials were incubated at 37°C for 48 h. The vials were agitated at 200 rpm. The fermentor was initially set at an agitation rate of 900 rpm and then lowered to 200 rpm after 2 h.

Hydrolysis experiments were performed in a similar manner without the addition of a fermentative microorganism.

Adsorption of Cellulase Enzymes

The adsorption of the enzymes onto various substrates was tested by incubating the crude enzyme mixture with the substrate for 10 min at a pH of 4.8 and a temperature of 28°C. After incubation the culture filtrate was assayed for endoglucanase, β-glucosidase, and filter paper activities and protein content. These values were compared to the activities of the crude enzyme mixture after incubation without the substrate.

RESULTS

The recycle of cellulase enzymes following a combined hydrolysis and fermentation procedure is dependent on several factors such as the thermal stability of the enzymes, the effect of ethanol on the enzymes and the adsorption of the enzymes onto the cellulose. The amount of recovery of the original enzyme considered to be necessary for an economically viable process is 60%.[2] In order to test the technical feasibility of this level of recovery, the activity of the cellulase enzymes remaining in the culture filtrate following the combined hydrolysis and fermentation process was studied.

The cellulase enzymes derived from the growth of the fungus *T. harzianum* E58 on cellulose were used for the hydrolysis of a 5% cellulose solution. The yeast *S. cerevisiae* was also added to the hydrolysis system so that the produced glucose would be quickly converted to ethanol and thus optimize the hydrolysis process. The glucose produced by the hydrolysis of cellulose is a known inhibitor of the cellulase enzyme system of *T. harzianum* E58, and its conversion to ethanol enhances the overall hydrolysis.[9] The ethanol produced during the CHF process is also inhibitory to the enzymes; however, the major effect of the ethanol is not directly on the enzyme activity but rather on the stability of the enzymes under the hydrolysis conditions.[10] This fact has direct implications for the recovery and recycle of the enzymes since the presence of ethanol will be detrimental to this procedure.

The combined hydrolysis and fermentation was performed in both small flasks and fermentors (Table I). Both systems produced similar levels of hydrolysis based on the production of ethanol and reducing sugars. The fermentor residue contained not only cellulose but also yeast cells from the fermentative aspect of the process.

Table I. Combined hydrolysis and fermentation of 5% commercial cellulose using enzyme system derived from growth of *T. harzianum* E58.[a]

Hydrolysis condition	Percentage of hydrolysis of cellulose	
	To reducing sugars	To glucose
Hydrolysis only[b]	52.8	45.5
CHF flasks	67.3	58.4
CHF fermentor	67.5	59.5

[a] Cellulase enzymes were used at 15 IU filter paper activity/g cellulose.

[b] Hydrolysis-only system means hydrolysis was performed at 45°C for 48 h, with no fermentative organisms added to the system. The flasks and fermentors were both combined hydrolysis and fermentation systems performed at 37°C.

Table II. Percentage of original enzyme activities measured in culture filtrate after CHF of 5% commercial cellulose and of control without added cellulose.

Hydrolysis conditions	Activity remaining in filtrate, % Endoglucanase	 β-Glucosidase	 Filter paper, 15 IU/g
Enzyme alone[a]	72.4	96.2	97.0
CHF flasks	58.5	116	65.8
CHF fermentor	49.0	101	61.3

[a] To assay for stability of enzymes, they were treated under identical conditions as CHF vials in absence of substrate.

Based on the nitrogen content of the residue, approximately 19% of the fermentor residue was yeast biomass.

The enzymes present in the culture filtrate were assayed in both the CHF systems and in flasks containing enzymes and no cellulose substrate. These flasks allowed the monitoring of the stability of the enzyme system under the CHF conditions. The results with the flasks containing enzymes with no added substrate (Table II) indicated that the endoglucanase component of the enzyme system was the least stable. Sixty percent of the filter paper activity was recovered from the CHF system, but only 50% of the original hydrolytic activity was present (Table III). Even the enzymes in the control, which had only been exposed to the CHF conditions and no substrate, produced 20% less glucose when they were subsequently used to hydrolyze cellulose. This result clearly indicated that even under ideal conditions 100% enzyme recycle is unrealistic. A more thermal stable enzyme system would obviously be more useful as even using CHF conditions with an incubation temperature of 37°C resulted in the loss of some enzyme activity.

Although the assayed enzyme activities differed for the flasks and the fermentor, the resulting products of the hydrolytic activity of the two enzyme preparations were similar. This again raises the question of the reliability of the assays for enzyme activities and points to the need for continual monitoring of the hydrolytic activity of the enzymes as the only means of realistically assessing the potential of a given enzyme preparation.

Table IV. Chemical composition of steam-treated water-extracted aspenwood (SEA-WI) before and after CHF process.[a]

Substrate	Hexosan, %	Lignin, %	Dry weight, g
Original SEA-WI	66.9	31.2	80
After CHF	36.9	52.7	47.4

[a] Dry weight of residue included dry weight of yeast biomass. Based on nitrogen content of residue, this amounted to 19% of residue weight. Percentage of hexosan and lignin was based on total dry weight of residue. Percentage of cellulose hydrolysis was 72.9.

The results indicated that the cellulose that remained following hydrolysis still exerted a strong adsorptive effect on the cellulase enzymes. The β-glucosidase component of the enzyme preparation was not adsorbed whereas the endoglucanase and exoglucanase components were strongly adsorbed by the unhydrolyzed cellulose. The activity of the β-glucosidase in the culture filtrate after adsorption was always found to be higher than the activity measured in the original filtrate. This apparent increase in activity may have resulted from the removal of the endo- and exoglucanase components of the enzyme system, which may block the access of the β-glucosidase to the cellobiose substrate. If we assume that approximately 70% of the cellulose was solubilized, the remaining 30%, or 1.5%, cellulose solution would adsorb the enzymes. Thus, the total percentage of hydrolysis was a major contributing factor to the recovery and recycle of enzymes. Once the base case with a commercial cellulose preparation was established, the feasibility of enzyme

Table III. Hydrolytic activity of culture filtrates derived from CHF of 5% cellulose and control without added cellulose.[a]

Hydrolysis conditions	Enzyme loading, FPU/g	Residual CHF products Residual sugars, mg/mL	 EtOH, mg/mL	Hydrolytic activity, % initial activity Reducing sugars	 Glucose
Initial	15	—	—	100	100
Enzyme alone	11.4	—	—	86.3	81.6
CHF flasks	7.8	3.7	9.2	51.6	49.2
CHF fermentors	6.9	3.0	9.4	50.9	46.4

[a] Culture filtrates from three systems added to 5% cellulose slurry. For this assay 1 g Solka Floc was mixed with 5 mL buffer and 15 mL culture filtrate incubated at 45°C for 24 h. Since culture filtrate taken from CHF systems contained both residual reducing sugars and ethanol, these products were added to test hydrolysis. Hydrolytic activity test was based on conversion of Solka Floc to glucose, and thus intermediate products of CHF had no bearing on calculations.

Table V. Recovery of cellulase enzymes in culture filtrate following CHF process of 5% steam-treated water-insoluble aspenwood.

	Enzyme activity, IU/mL			
	Endoglucanase	β-Glucosidase	Filter paper	Protein, mg/mL
Original activity	15.1	0.76	0.75	0.86
Recovered activity	1.9	0.39	0.12	0.17

recycle was investigated using pretreated lignocellulosic material. The pretreatment procedure currently used as a base case involves the steam treatment of aspenwood at 240°C for 80 s and subsequent removal of soluble pentosans and inhibitors with a simple water extraction procedure. This material, which is termed *steam-exploded aspenwood–water insoluble (SEA-WI)* was used at a 5% dry-weight concentration in a CHF process. The chemical composition of the lignocellulosic material before and after hydrolysis is given in Table IV. The reduction in dry weight was estimated to be about 59% of the original; however, due to the presence of the yeast biomass, the percentage of loss of the cellulose component was actually 73%. After the hydrolysis there still remained a 1% cellulose suspension in the fermentor.

The assayed enzyme activity of the culture filtrate before and after the CHF process is given in Table V. The filter paper activity of the cellulase enzymes recovered from the process was less than 20% of the original filter paper activity. This low enzyme recovery could not be accounted for by the unhydrolyzed cellulose portion. Other components of the steamed material must therefore be affecting the release of the enzyme following hydrolysis.

The hydrolytic activity of the recovered enzymes was monitored and is reported in Table VI. This table also illustrates the hydrolytic activity of the recovered enzymes when they were combined with various proportions of fresh enzymes. These results indicated that with the present process conditions the recycle of enzymes does not appear beneficial.

Although the hydrolysis of the commercial cellulose was the same as that obtained with the lignocellulosic residue, the recovery of the enzymes was very different. The major difference between these substrates was the amount of lignin present in the SEA-WI substrate. Lignin constituted 31% of the original substrate and as much as 51% of the final posthydrolysis residue. Several researchers have reported[11,12] that lignin strongly adsorbs the cellulase enzymes. This possibility was further investigated.

The adsorption of the cellulase enzymes onto different lignin fractions were monitored under conditions similar to those found in a CHF process. The results presented in Table VII indicated that the lignin did not adsorb the cellulase enzymes. However, when the adsorption of cellulase enzymes to various pretreated lignocellulosic residues was compared (Table VIII), the results indicated that some sort of adsorption was occurring with the lignin–cellulose complex. Although lignin itself did not appear to adsorb cellulase enzymes, the presence of residual lignin in the lignocellulosic residue appeared to severely affect the recovery of the cellulase enzymes.

In our process design the removal of lignin before or after the CHF process is still one of the decisions that must be finalized. Although the presence of lignin did not appear to affect the cellulose hydrolysis,[13] the presence of lignin did influence the recycle of cellulase enzymes. We next monitored the recovery of cellulase enzymes after a CHF process on delignified aspenwood. The substrate that was chosen for this purpose was steamed aspenwood that had been water and alkali

Table VI. Hydrolytic activity of various proportions of recovered and fresh enzymes.[a]

	Enzyme profile[b]			Hydrolysis products	
Original/recovered filtrate, %	Endoglucanase	β-Glucosidase	Filter paper	Reducing Sugars, mg/mL	Glucose, mg/mL
0/100	30.0	6.0	2.0	5.7	3.1
20/80	94.8	7.9	4.6	16.0	11.3
40/60	212.0	13.0	9.6	21.0	15.6
60/40	299.2	15.6	13.0	25.2	19.3
80/20	385.6	18.0	16.5	27.9	22.1
100/0	472.0	20.6	20.0	30.8	25.4

[a] Original culture filtrate had enzyme activity of 23.6 IU endoglucanase, 1.03 IU β-glucosidase, and 1.00 IU filter paper activity. Hydrolysis was performed at 5% cellulose concentration for 48 h at 45°C.

[b] Enzyme profile given as IU/g substrate.

Table VII. Adsorption of cellulolytic enzymes to various lignin samples and to pretreated lignocellulosic substrates.

Substrate, 5%	Enzyme remaining in filtrate, % Endoglucanase	 β-Glucosidase	 Filter paper	Protein in filtrate, %
Solka Floc	30.5	117.0	32.5	34.8
SEA-WI[a]	11.8	67.7	13.8	26.7
Lignin A[b]	103.6	100.0	97.1	97.6
Lignin B[c]	96.3	103.4	90.0	85.9
Lignin C[d]	100.9	103.4	100.0	96.4
Xylan	105.7	113.3	108.6	120.8
SEA-WI (enz)[e]	66.7	85.0	56.4	67.9

[a] Steam-treated aspenwood heated at 240°C for 180 s and then water extracted.

[b] Derived from steamed aspenwood treated for 220 s at 240°C. Lignin was solubilized with methanol, then filtered and reprecipitated with water. Approximately 50% original lignin recovered by this procedure.

[c] Derived from aspenwood ball-milled for 2 weeks and then partitioned. Material dissolved in 90% acetic acid to remove carbohydrate contaminants.

[d] Derived from steam-treated aspenwood treated at 240°C for 80 s. Lignin extracted with 0.4% sodium hydroxide.

[e] Same SEA-WI sample as described above enzymatically hydrolyzed by CHF process. Original cellulose content was 57%, and after CHF cellulose content was between 3 and 10%.

extracted and is termed *steam-exploded aspenwood–water and alkali insolubles (SEA-WAI)*. The alkali extraction removed 75% of the lignin, as can be seen from the data in Table VIII. The lignin composition of the material after hydrolysis was 17%, which was equivalent to less than half of the total residue remaining after the CHF of the SEA-WI substrate.

The recovery of the cellulase enzymes after the CHF of the water- and alkali-extracted aspenwood (SEA-WAI) is reported in Table IX, and the results were found to be as poor as with the substrate with a higher lignin content.

The results indicated that for successful recycle of enzymes to occur, the percentage of hydrolysis of cellulose must be at a high enough level so that little cellulose remains in the residual mixture to adsorb the cellulase enzymes. The high conversion of cellulose to sugars will require more research into the mode of action of the cellulase enzymes and into the pretreatment process so that more cellulose is accessible to the cellulase enzymes.

Although the recycle of enzymes is an economically attractive process, our results have shown that with the present technology this concept is not worthwhile. Another area of recycle that will also have an impact on the economic feasibility of the process is the recycle of the unhydrolyzed lignocellulosic residue. Since the complete hydrolysis of the cellulose appears to be unrealistic with our present technology, we have investigated the possibility of using the unhydrolyzed portion of the cellulose as the growth substrate for the production of cellulase enzymes.

It was hoped that the recalcitrant nature of the cellulose remaining after cellulose hydrolysis might cause the production of a more active enzyme since the physical characteristics of the cellulose substrate is believed to influence the enzyme profile produced.[14] The recalcitrant cellulose was produced by performing a CHF process on Solka Floc and using the residual cellulose–yeast mixture as the substrate for the growth of

Table VIII. Chemical compositon of steam-treated water- and alkali-extracted (SEA-WAI) aspenwood before and after enzymatic hydrolysis.[a]

Substrate	Hexosan %	Lignin %	Dry weight g
Original (SEA-WAI)	90.0	8.2	80
After hydrolysis	61.6	17.2	38.3[b]

[a] Enzymatic hydrolysis accomplished by CHF process described in Materials and Methods.

[b] Dry weight of residue included dry weight of yeast biomass. Based on nitrogen content of residue, this amounted to 19% of residue weight. Percentage of hexosan and lignin based on total dry weight of residue. Percentage of cellulose hydrolysis was 67.2.

Table IX. Recovery of cellulase enzymes in culture filtrate following CHF process of steam-treated water- and alkali-insoluble aspenwood.

Enzyme activity, IU/mL	Before CHF	After CHF
Endoglucanase	14.6	1.6
β-Glucosidase	0.83	0.16
Filter paper	0.75	0.09
Protein mg/mL	0.96	0.10

Table X. Production of cellulase enzymes from growth of *T. harzianum* E58 on spent cellulose.[a]

Substrate, 1%	Nitrogen Supplement, 0.1%	Enzyme activity of filtrate, IU/mL			Protein, mg/mL
		Endoglucanase	β-Glucosidase	Filter paper	
Solka Floc	BP	20.6	0.92	1.26	1.28
	YE	19.0	0.78	1.14	1.22
Spent SF	BP	34.3	1.26	1.99	1.95
	ND	28.4	0.98	1.47	1.59

[a] Spent SF was residual cellulose remaining after CHF of 5% Solka Floc. Percentage of hydrolysis was 76. Cellulose dried following CHF process, and nitrogen content measured. Abbreviations: BP, Bacto peptone; YE, yeast extract; ND, from nitrogen determination of cellulose 0.23% yeast extract present.

the fungus *T. harzianum* E58. The results are presented in Table X. The media for the Solka Floc controls contained either peptone or yeast extract because the production of enzymes may have been influenced by the yeast biomass contained in the cellulose residue from the CHF process. Previous results[12] have indicated that the presence of yeast extract is inhibitory to the action of the enzymes. The addition of yeast extract to the Solka Floc control did not appreciably affect the production of the cellulase enzymes. When spent cellulose was used as the growth substrate, higher levels of the cellulase enzymes could be found in the culture filtrate.

The hydrolytic activity of the produced culture filtrates described in the previous table were assayed (Table XI). The culture filtrate originating from the growth of the fungus on the spent cellulose was marginally better than the culture filtrate derived from the control cellulose. These results are very encouraging as they offer another avenue for the reduction of the costs involved in the enzyme production step.

DISCUSSION

The recovery of cellulolytic enzymes derived from the fungus *T. harzianum* E58 from the hydrolysis mixtures containing lignocellulosic material was less than 20%. This low value has also been reported by Puls,[14] who used steamed birchwood as the substrate and cellulase enzymes derived from *T. reesei*. The removal of the lignin component prior to the hydrolysis step did not improve the poor recovery of the enzymes. Although lignin was shown not to adsorb the cellulolytic enzymes, the presence of this material in the starting substrate did influence adsorption. The lack of enzyme adsorption onto lignin is contradictory to results obtained by Klyosov (personal communication). The difference in the results could be explained by a difference in scale of the experimental procedure. The adsorption procedure reported here was designed to test the effect of lignin in a system similar to the CHF process. The ratio of lignin to enzymes was 15 IU filter paper activity/g lignin. At this level adsorption could not be detected. The research protocol used by other workers used enzyme levels 20 times lower than ours, which may explain the difference in the results.

Table XI. Hydrolytic activity of culture filtrates described in Table X.[a]

Culture filtrate	Hydrolysis, %	
	To reducing sugars	To glucose
Solka Floc (BP)	55.4	40.8
Solka Floc (YE)	53.8	39.6
Spent SF (BP)	56.9	43.4
Spent SF (ND)	53.7	40.6

[a] Hydrolysis performed on 5% Solka Floc with 15 mL culture filtrate/g cellulose. Incubation time 48 h.

The combination of the lignin and cellulose was found to have a strong adsorptive effect on the cellulase enzymes. In fact, this combination adsorbed the β-glucosidase component of the cellulase system, whereas commercial cellulose alone did not. This result indicates that the phenomenon we observed was not solely due to adsorption and that inhibition of the enzyme activity may have played a role.

From this data it was apparent that the benefit of recycling cellulolytic enzymes will primarily depend on the achievement of high levels of hydrolysis. The complete hydrolysis of cellulose will require more research into the mode of action of the cellulase complex and the pretreatment of the lignocellulosic residue.

An alternative form of recycle that may offer some reductions in the overall cost of the production of the cellulase enzymes is the reuse of the recalcitrant cellulose remaining after the hydrolysis material. This approach was found to be feasible with the commercial cellulose Solka Floc. Future research will now focus on applying this concept to the lignocellulosic residues remaining in the fermentor.

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ENZYMATIC CONVERSION OF LIGNOCELLULOSIC MATERIAL TO FUELS

M. Mes-Hartree, C. Hogan, G. Louis-Seize,
Q. Nguyen and J.N. Saddler. 1987
6th Canadian Bioenergy, R&D Seminar

ENZYMATIC CONVERSION OF LIGNOCELLULOSIC MATERIAL TO FUELS

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The technical and economic feasibility of the commercial production of liquid fuels from aspenwood was studied. The conversion process relied on steam pretreatment, fractionation and enzymatic hydrolysis of the carbohydrate polymers to monomeric sugars. The sugars were fermented to ethanol or butanediol.

Les possibilités techniques et économiques d'une production commerciale de carburants à partir de bois de tremble ont été étudiées. Le procédé de conversion se fonde sur un prétraitement à la vapeur, un fractionnement et une hydrolyse enzymatique des polymères d'hydrates de carbone en sucres monomères. Les sucres sont transformés en éthanol ou en butanediol par fermentation.

INTRODUCTION

The process under study is outlined in Figure 1. The first step in the process was the steam pretreatment of aspenwood chips which are then water and caustic extracted. The water extraction step removes the majority of the hemicellulose component of the wood while the caustic extraction removes a large percentage of the lignin. The residual material contained over 90% cellulose and was used for the production of the cellulase enzymes and for hydrolysis to glucose which was fermented to ethanol.

The feedstock used for enzyme production must be cheap as this step represents a large part of the overall costs involved with the process (Isaacs, 1984, Nystrom et al, 1984). In our process the cellulases enzymes were produced by the fungus Trichoderma harzianum E58 during growth on steamed aspenwood.

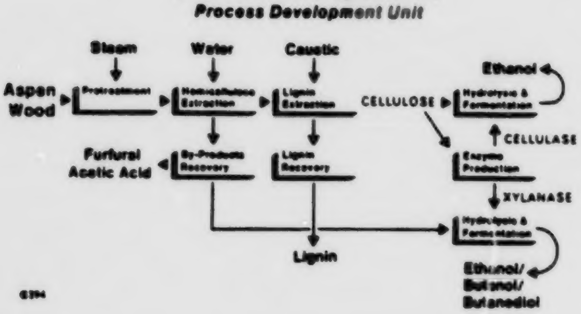
Simplified Flow Diagram of the Process Development Unit
Steam
Water
Caustic
Aspen Wood
Pretreatment
Hemicellulose Extraction
Lignin Extraction
CELLULOSE
Hydrolysis & Fermentation
Ethanol
CELLULASE
Furfural Acetic Acid
By-Products Recovery
Lignin Recovery
Enzyme Production
XYLANASE
Hydrolysis & Fermentation
Lignin
Ethanol/ Butanol/ Butanediol
6394

Figure 1. Simplified process design

The scale up of the enzyme production using steamed wood as the carbon source will cause material handling problems. It is for this reason that several plant designs have relied on a soluble carbon source such as lactose for enzyme production even though lactose induces less enzymes (Warzywoda et al, 1983). The use of lactose also limits the number of plant locations since a plant would have to be located in a region where both forestry residues and cheese whey accumulate. If one considers the strides being made in the conversion of cheese whey to industrial/potable ethanol, single cell protein and baker's yeast (Russell, 1986), cheese whey may not in the future be a readily available waste material.

RESULTS

The use of the lignocellulosic feedstock for both enzyme production and hydrolysis will require cellulose to be split between these two activities. Other researchers (Chahal et al, 1982) have indicated that approximately 10% of the incoming feedstock is required for enzyme production purposes. The amount of feedstock required for enzyme production will depend on both the enzyme loading (amount of enzyme used in hydrolysis reactor) and the enzyme yield (amount of enzyme produced in the production reactor).

The enzyme loading in the hydrolysis step has a major impact on the glucose yield (Figure 2). There was no linear relationship between enzyme loading and glucose production, and higher enzyme loadings produced smaller increments in glucose yield. High enzyme loadings required greater amounts of enzyme, which caused more of the pretreated aspenwood to be diverted from glucose production to enzyme production.

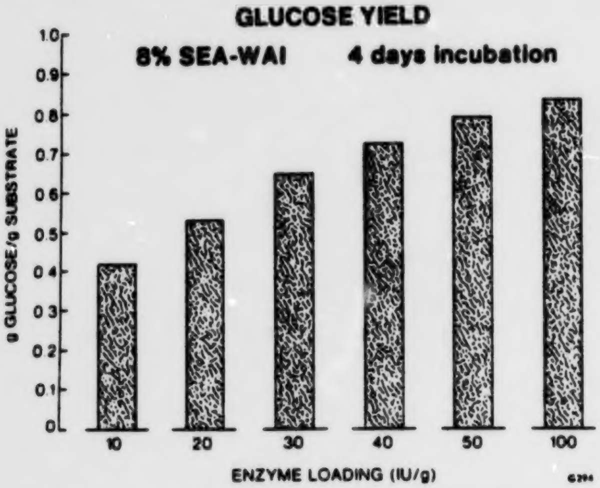

Figure 2. Production of glucose from steamed aspenwood using various enzyme loadings. The enzymes were derived from the growth of T. harzianum E58 on steamed aspenwood. The substrate concentration was 8%, incubation time, 4 days and incubation temperature 45°C. SEA-WAI-aspenwood steamed at 240°C for 80 secs; water and alkali extracted.

The effects of enzyme yield and enzyme loading on glucose production were calculated for the standard process; splitting the steamed aspenwood between enzyme production and hydrolysis as required by each process (Figure 3). It was seen that an increase in enzyme yield resulted in a substantial increase in glucose production except at the high enzyme loading of 100 IU/g substrate which resulted in a decrease in glucose production due to the substrate requirements for enzyme production. An enzyme loading of 50 IU/g substrate appeared to be the optimum enzyme loading for our conditions.

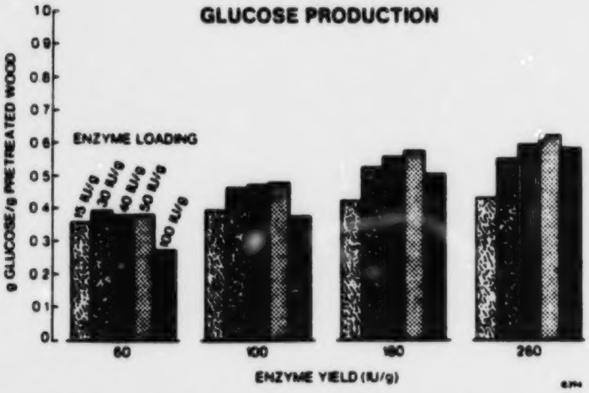

Figure 3. The effect of enzymes loading and enzyme yield on the production of glucose from the steamed aspenwood.

The present enzyme yield achieved with T. harzianum grown on steamed aspenwood at a high substrate concentration was about 60 IU/g substrate. Increasing this yield to 260 IU/g substrate would have a significant impact on glucose production. We have been able to achieve enzyme yields of 260 IU/g at substrate concentrations of less than 1%. The enzyme yield decreased dramatically as the substrate concentration was increased. This decline in enzyme yield was partially attributed to the repression of cellulase expression at high substrate concentrations due to release of glucose. This problem could be overcome with the isolation of a catabolite derepressed mutant that would be unaffected by the presence of glucose.

Even at the high enzyme yield of 260 IU/g, the glucose production of 0.65 g/g steamed aspenwood was low. This low glucose production is caused in part by the recalcitrant nature of the cellulose and by the low specific activity of the enzymes. We investigated the potential gains in glucose production which might be achieved by two modifications of the hydrolysis portion of the process (Figure 4). The first option was recycle of the enzymes after the hydrolysis process and the second option was the recycle of the unhydrolysed cellulose to the enzyme production stage. The impact of these options on glucose production are presented in Figure 5.

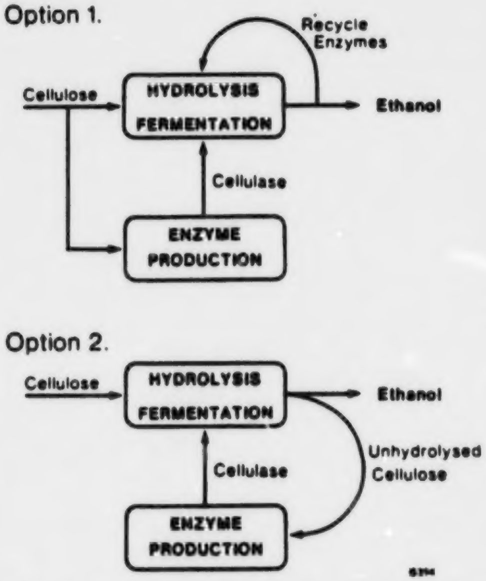

Figure 4. Two process options which can increase the production of glucose from steamed aspenwood.

Option 1 - Recycle of enzymes following hydrolysis

Option 2 - Recycle of unhydrolysed cellulose from hydrolysis to enzyme production

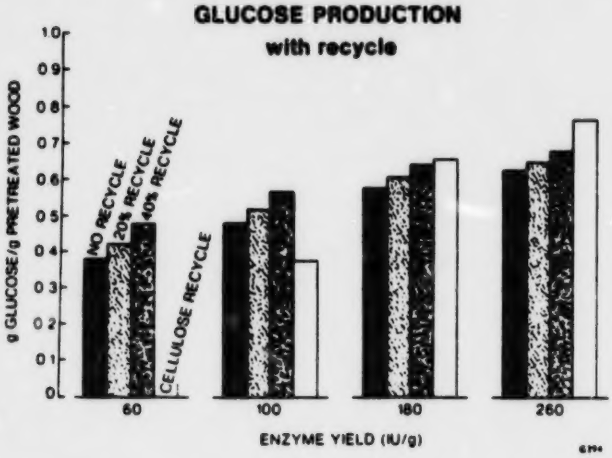

Figure 5. The effect of enzyme recycle or cellulase recycle on glucose production at various enzyme yields. The enzyme loading was constant at 50 IU/g substrate.

The two levels of enzyme recycle chosen for these calculations were 20 and 40% since our previous results had indicated that this level of recycle was achievable under our current process conditions (Mes-Hartree et al, 1937). Even this low level of enzyme recycle had a beneficial impact on glucose production. Obviously the cost of the recycle will also have to be considered before this option is incorporated into the process, however, the beneficial effect of enzyme recycle on glucose production warrants a further study.

At the higher enzyme yields of 180 and 260 IU/g cellulose, the biggest impact on glucose production was when cellulose was recycled. We have tested the feasibility of such a recycle scheme using pure cellulose as the substrate. We found that both the quantity and the quality of the enzymes produced from the recycled cellulose were superior to the enzymes produced from the unhydrolysed cellulose. The presence of lignin in the steamed aspenwood may however, interfere with this scheme and research should focus on this area since it would have a marked impact on glucose production.

SUMMARY

The mass balance analysis of the hydrolysis and enzyme production steps of the process have indicated three areas in the enzyme production step which impact on the production of glucose. These areas were enzyme yield, recycle of enzyme from the hydrolysis reactor and recycle of unhydrolysed cellulose from the hydrolysis reactor to the fermentor reactor.

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INFLUENCE OF GROWTH SUBSTRATE ON PRODUCTION OF CELLULASE ENZYMES BY
TRICHODERMA HARZIANUM E58
M. Mes-Hartree, C.M. Hogan and J.N. Saddler, 1988
Biotechnol. Bioeng. 31:725-729

Influence of Growth Substrate on Production of Cellulase Enzymes by *Trichoderma harzianum* E58

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Cellulase production by *Trichoderma harzianum* E58 grown on lactose and various cellulosic substrates such as Solka Floc, Avicel, and steamed aspenwood was investigated. The culture filtrates of *T. harzianum* E58 obtained after growth on these substrates were assayed for their cellulase activities and overall hydrolytic activities. The severity of the steaming conditions used for the aspenwood had a pronounced effect on the cellulolytic activity of the produced culture filtrates. Those substrates that were more readily hydrolyzed by the cellulase complex were the poorest substrates for inducing an active cellulase complex. Substrates such as acid-impregnated aspenwood and lactose induced a less hydrolytic efficient cellulase complex than more recalcitrant substrates such as microcrystalline cellulose.

INTRODUCTION

The enzymatic conversion of lignocellulosic residues to fuels and chemicals requires large amounts of cellulase enzymes for the hydrolysis of cellulose to glucose. The structural integrity of lignocellulosic material is such that a pretreatment of the material is necessary before the enzymatic conversion can be attempted. Pretreatment by steaming has been shown to render the cellulose in the lignocellulosic residue more accessible to the cellulase enzymes.[1]

The production of cellulase enzymes should be an integral part of any enzymatic process that converts lignocellulosic material to sugars. The feedstock used for the production of enzymes must be cheap, as the cost of enzyme production represents a large part of the overall costs of producing sugars from lignocellulosic material.[2] The designs of many conversion processes rely on lactose as the carbon source even though less enzymes are produced on lactose.[2–4] An alternative approach is to utilize the incoming feedstock for enzyme production. Various researchers[5,6] have successfully utilized steam-treated wood as the growth substrate for enzyme production.

The steaming process has been optimized for the hydrolysis component of the process, but little work has been done on the effect of various steaming conditions on enzyme production. The production of enzymes may be influenced by a number of factors, such as the growth of the fungus, the induction of the enzyme, and catabolite repression. In this article we describe our investigations into the effect various steaming conditions applied to aspenwood have on cellulase enzyme production by the fungus *Trichoderma harzianum* E58.

MATERIALS AND METHODS

Growth and Enzyme Production

The fungus *Trichoderma harzianum* E58 was taken from the Forintek Culture Collection. A spore inoculum was used to initiate growth in shake flasks containing Vogel's medium and 2% glucose. After 2 days at 28°C a mycelium suspension was used to initiate growth in 500-mL Erlenmeyer flasks containing a 1% concentration of cellulosic material or soluble sugars in 150 mL medium. The flasks were incubated for 3–4 days at 28°C with an agitation rate of 150 rpm. The culture was harvested by sequential filtration through a Whatman glass microfibre filter and a Nalgene sterilization unit with a pore size of 0.45 μm.

The cellulase preparation used for the accessibility assay was produced in a 28-L New Brunswick fermentor with a working volume of 20 L. The culture filtrate was concentrated by ultrafiltration using a Pellicon cassette system (Millipore) with a molecular weight cut-off of 10,000 daltons. The resulting concentrated filtrate was lyophilized and stored at 4°C.

Assays

Total reducing sugars were estimated using the dinitrosalicylic acid assay.[7] Glucose was determined by the glucostat enzyme assay.[8] Enzymatic activity was determined by the method of Mandels.[9] The standard hydrolysis assays were performed under aseptic conditions on cellulosic material that had been autoclaved before use.

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Accessibility Assay

A standard hydrolysis test was performed in 50-mL Erlenmeyer flasks (reaction volume 20 mL) with a set enzyme loading of 15 FPU/g substrate (dry weight). The hydrolysis was performed at a 5% substrate concentration (dry weight) in sodium acetate buffer at pH 4.8, 45°C, for 24 h, with shaking at 150 rpm. After the incubation period, the contents of the flasks were centrifuged at 6,000 rpm for 10 min and the supernatant fluid analyzed for reducing sugars and glucose. The results of the test are reported as grams of sugars per gram of cellulose.

Hydrolysis Assay

This assay was similar to the one described in the preceding assay. In this case we used a constant substrate and a variable enzyme preparation. The commercial cellulose Solka Floc was used at a 5% concentration. All other conditions were identical to the accessibility assay. The enzyme preparations were added as 75% of the final volume.

Steam Pretreatment

Steam pretreatment was performed as previously described.[1] In some cases the aspenwood chips were vacuum impregnated with 0.2% sulfuric acid for 64 h at 50°C and blotted dry between towels. These chips were steamed at 220°C or 240°C. All of the pretreated samples were water extracted at a 5% slurry concentration, stirred for 60 min, filtered on a 70-μm expanded polyethylene filter, resuspended in fresh water at 5% concentration, stirred for 30 min, and filtered prior to being used in the experiments.

Commercial Cellulose

Solka Floc (Brown and Co., Hackensack, NJ) was made from hammer-milled wood pulp and was a fibrous grade of cellulose. Two grades of this material were used, BW300 and SW40. The SW40 grade was coarser than the BW300 grade, which had been ball milled to pass 300 mesh.

Avicel pH 101 (FMC Corp., Rockland, ME) is a microcrystalline cellulose derived from fibrous plants. It is a particulate free-flowing powder.

The CF 11 cellulose powder was a Whatman medium-length fiber.

Solka Floc AS1040 (Brown and Co.) was made from purified cotton linters.

Crystallinity Index

The crystallinity index (CrI) was determined using an x-ray diffractometer by the method of Segal et al.[10]

RESULTS AND DISCUSSION

The fungus *T. harzianum* E58 produces extracellular cellulase enzymes that can degrade crystalline cellulose.[11] The enzyme mixture also contains high levels of B-glucosidase and xylanase activities. Cellulase production is under regulatory control, with catabolite repression and active induction both playing a role. In order to further elucidate the induction of cellulase enzymes, various soluble sugars such as glucose, cellobiose, maltose, sucrose, glycerol, and starch were tested as substrates for growth and enzyme production in *T. harzianum*. While the fungus was able to utilize all the sugars for growth, no cellulase enzymes were produced. Lactose was also used in the preceding experiment, and was the only soluble sugar capable of inducing cellulase synthesis (Fig. 1). Endoglucanase and filter paper activity first appeared in the culture filtrate at 48 h, with maximum production occurring at 48–72 h. This time period corresponded to the maximum growth period.

Evidence in the literature suggests that the nature of the carbon source plays a strong role in cellulase production.[12-14] Solubilization of amorphous cellulose can be accomplished by either endoglucanase or exoglucanase independently, while hydrolysis of crystalline cellulose requires the synergistic interaction of both endo- and exoglucanases.[12] The nature of the carbon source used to induce the endoglucanase component has been shown to influence the hydrolytic activity of the enzyme preparation.[12] Thus, a close relationship exists between the substrate used for induction of the cellulase enzymes and the efficiency of the produced enzymes to degrade crystalline cellulose.

A comparison of the quality of enzymes produced on soluble and insoluble substrates was made. Cellulase enzymes were produced using various commercial cellulose preparations or lactose as the inducing substrate, and the cellulase profile of each preparation was determined (Table I). The rapid assay methods used to determine the enzyme profile do not give an accurate assessment of the hydrolytic potential of an enzyme preparation under use conditions. To accomplish this, the enzyme preparations were used in a standard hydrolysis test. As there was a wide variation in the activity of the enzyme preparations that would have obscured a comparison of their relative efficiencies, the enzyme preparations were diluted to a con-

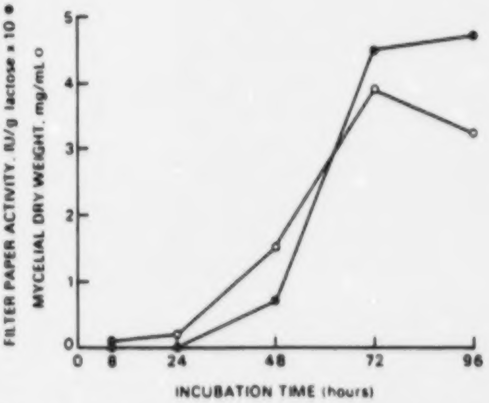

Figure 1. Growth and cellulase production of *T. harzianum* E58 on 0.5% lactose.

Table I. Production of extracellular cellulase enzymes from growth of *T. harzianum* on various commercial celluloses and lactose.[a]

Commercial cellulose	Enzyme activities (IU/g substrate)		
	Endoglucanase	β-glucosidase	Filter paper
Solka Floc BW300	1600	51	103
Solka Floc SW40	2970	111	166
Avicel pH 101	3250	118	173
Solka Floc AS 1040	2050	95	101
CF11	150	13	8
Lactose	360	22	27

[a] Fungus was grown on 1% substrate concentration for 4 days at 28°C.

tant filter paper activity prior to being used to hydrolyze Solka Floc BW300 and SW40. The crystallinity and accessibility of the growth substrates used to produce the enzyme complex was also determined (Table II). There was no obvious relationship between the accessibility or the crystallinity of the growth substrate and the production of the cellulase enzymes.

The cellulose that induced the most efficient enzyme system was Avicel. The least amount of enzyme was produced from growth on CF11, and this was due to poor growth of the fungus on this highly crystalline substrate. The least efficient enzyme preparation was derived from the growth of the fungus on lactose. This enzyme preparation produced 25% less sugars than enzymes derived from the commercial cellulose, Solka Floc. This point is worth considering in the design of a commercial process if cheese whey is being considered as the feedstock for enzyme production. The preliminary economic projections[3] for this approach have indicated that there may be problems in locating a lignocellulose plant in relation to both a dairy-processing plant and the wood residues. The preferred carbon source for the induction of the cellulase enzymes would appear to be the pretreated lignocellulosic material used for hydrolysis.

Steam pretreatment has been found to be an effective method of increasing the accessibility of lignocellulosics to enzymatic attack.[1] Aspenwood chips were steamed under a wide range of steaming conditions, and the water-washed material was used as the carbon source for the growth of the fungus *T. harzianum* E58 (Table III). The enzyme profiles were reported on a gram cellulose basis for comparative purposes because the percentage of cellulose present in each of the pretreated wood samples was different.

The enzyme activity profile of the culture filtrates provides an estimation of the potency of a given enzyme preparation. However, the actual hydrolytic activity of an enzyme can only be assessed under use conditions. A standard hydrolysis of commercial cellulose was performed using the culture filtrates described in Table III. The results from this hydrolysis are reported in Table IV. The cellulase enzymes produced from wood steamed at 240°C for between 80 and 240 s had greater hydrolytic activity than cellulase enzymes produced from acid-impregnated wood.

The pretreated substrates examined for enzyme production were also used to determine the optimal steaming conditions for hydrolytic purposes. The effectiveness of pretreatment was determined by measuring the accessibility of the substrate to the enzyme. Accessibility is measured by a standard hydrolysis test where the substrate is present at a 5% (dry-weight) substrate concentration. Other parameters such as time, enzyme loading, and temperature are kept constant. This test was used for comparative purposes, and the results are not those obtained under optimum conditions.

A standard enzyme preparation from *T. harzianum* E58 was used to hydrolyze the various steamed aspenwood samples described in Tables III and IV, and these accessibility values are reported in terms of the amount of reducing sugars released per gram of original cellulose. The total units of filter paper produced by the aspenwood following each steaming condition and the accessibility of this material to cellulolytic enzymes are plotted in Figure 2. These re-

Table II. Hydrolytic activity of extracellular enzymes derived from growth of *T. harzianum* on various commercial celluloses and lactose.

Substrate (for enzyme production)[a]			Hydrolytic activity (g/cellulose/24 h)[b]			
			Solka Floc BW300		Solka Floc SW40	
Name	CrI	Accessibility	Red sugars	Glucose	Red sugars	Glucose
Solka Floc BW300	71.2	0.311	0.301	0.195	0.223	0.168
Solka Floc SW40	85.7	0.286	0.281	0.198	0.242	0.183
Avicel	89.2	0.254	0.311	0.216	0.286	0.217
Solka Floc AS1040	89.5	0.244	0.259	0.192	0.230	0.180
CF11[c]	95.2	0.124	0.099	0.073	0.049	0.044
Lactose	—	—	0.223	0.167	0.190	0.151

[a] Growth substrate used at 1% concentration. CrI refers to crystallinity index. Accessibility is g reducing sugars/g cellulose released after 24 h hydrolysis under standard conditions (see Materials and Methods).

[b] Hydrolytic activity of culture filtrate determined using either 5% Solka Floc BW300 or SW40 as substrate.

[c] Culture filtrates diluted to standard activity of 4 IU filter paper/g substrate except for CF 11, which undiluted was at 1.6 IU/g substrate.

Table III. Production of extracellular cellulase enzymes from growth of *T. harzianum* on various steam-treated aspenwood samples.[a]

Steam conditions			Enzyme activities (IU/g cellulose)		
0.2% H_2SO_4	Temperature (°C)	Time (s)	Endoglucanase	β-Glucosidase	Filter paper
–	240	20	1615	60	68
		40	3102	70	105
		80	5317	117	193
		120	4984	116	188
		180	5917	149	261
		240	5450	135	202
+	220	10	2255	83	106
		20	1320	112	73
		40	1696	92	80
		80	1250	94	80
+	240	10	1076	72	60
		20	608	77	48
		40	541	71	43

[a] Fungus grown on 1% substrate concentration (dry weight) for period of 4 days at 28°C.

Table IV. Hydrolytic activity of extracellular cellulase enzymes derived from growth of *T. harzianum* on various steam-treated aspenwood samples.[a]

Steam conditions			Hydrolytic activity (g/g cellulose)	
0.2% H_2SO_4	Temperature (°C)	Time (s)	Reducing sugars	Glucose
–	240	20	0.324	0.234
		40	0.408	0.300
		80	0.494	0.371
		120	0.492	0.377
		180	0.512	0.396
		240	0.484	0.376
+	220	10	0.430	0.326
		20	0.366	0.268
		40	0.374	0.276
		80	0.368	0.264
+	240	10	0.346	0.246
		20	0.294	0.214
		40	0.284	0.200
Solka Floc (BW 300)			0.518	0.392

[a] Hydrolysis performed on 5% Solka Floc (BW 300) for 24 h at 45°C with shaking. Culture filtrates described in Table III added on 75% (v/v) basis.

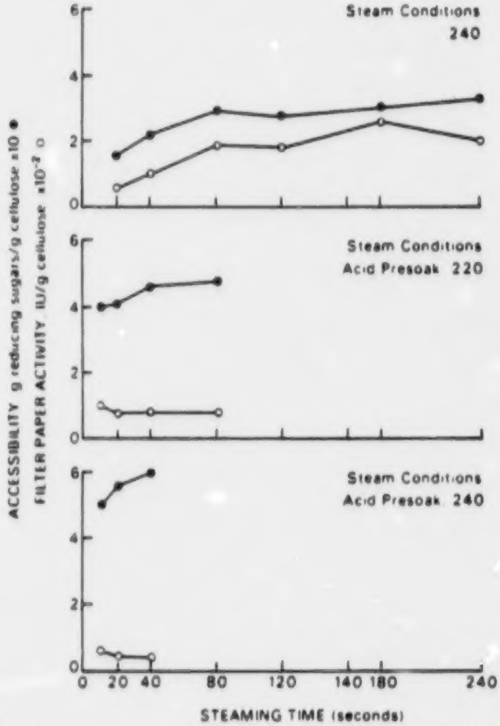

Figure 2. Production of extracellular cellulase from growth of *T. harzianum* E58 on various pretreated aspenwood samples and accessibility of these samples to cellulase complex.

sults indicated that lower enzyme levels were produced from the more readily hydrolyzed substrates such as the acid-impregnated steamed aspenwood.

Some of the factors that may be important in the production of extracellular enzymes are the growth of the fungus, the induction of the enzymes, and catabolite repression. Poor growth may be the reason for the low enzyme production from the steaming conditions at 240°C for 20 or 40 s. These conditions had lower accessibility numbers and were not as severely treated as the other samples. Enzyme production appeared to be a primary metabolite, as enzymes were produced at a maximal rate during exponential growth phase (Fig. 1). Although carbon-limited conditions appear to stimulate enzyme production,[15] there must be sufficient carbon to support fungal growth.

Previous workers have noted that a definite relationship exists between crystallinity and the rate or degree of enzymatic hydrolysis.[16] The rate at which cellulose is degraded influences the rate of formation of the inducer and also of the soluble catabolite repressors.[17] An amorphous or accessible substrate is readily degraded to glucose, and catabolite repression of cellulase synthesis occurs to a greater degree than with a slower rate of glucose liberation. Consequently, there must be an optimum rate of cellulose degradation that supports growth and allows for a slow feed of glucose, thereby avoiding or reducing the effects of catabolite repression.

The actual mechanism of cellulase regulation in filamentous fungi has yet to be fully resolved. It has been suggested that the induction of cellulase enzymes is triggered by some soluble hydrolysis product derived from the breakdown of cellulose.[18] However, it is difficult to imagine how this proposal could explain the effect the physical characteristics of the cellulose had on the induction of the cellulase complex. Binder and Ghose have suggested[19] that induction of a complete cellulase system requires physical contact between the mycelium and the cellulose, and this may explain the effect the physical characteristics of the cellulose have on the induction of the cellulase complex.

The steam-treated aspenwood was shown to be a suitable carbon source for the production of cellulase enzymes from the growth of the fungus *T. harzianum* E58. The optimal steaming condition for the purpose of enzyme production was found to be 240°C for 180, s and this was different from the optimal steaming conditions for hydrolysis purposes, which involved the use of an acid catalyst.

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RECYCLE OF CELLULASES AND LIGNOCELLULOSIC RESIDUE AFTER HYDROLYSIS OF STEAM-PRETREATED ASPENWOOD

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RECYCLE OF CELLULASES FOR HYDROLYSIS AND LIGNOCELLULOSIC RESIDUE FOR ENZYME PRODUCTION AFTER HYDROLYSIS OF STEAM-PRETREATED ASPENWOOD

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A 10% concentration (w/v) of steam-exploded, water-and-alkali extracted aspenwood was hydrolyzed with Trichoderma harzianum E58 cellulases using various modes of substrate and enzyme addition. Although cellulose conversion was high, enzyme recovery was low in all cases. Low enzyme recovery was due to a combination of thermal inactivation and adsorption of the cellulases onto the lignocellulosic residue. Enzyme recycle did not appear feasible as the activity of the recovered cellulases towards crystalline cellulose was low. The residual material from enzyme hydrolysis was found to be a suitable carbon source for cellulase enzyme production by T. harzianum based on enzyme yield and hydrolytic potential. These residues could only be used at a 1% concentration, since at higher substrate loadings cellulase production was reduced, likely due to lignin inhibitors.

INTRODUCTION

The commercial production of ethanol from lignocellulosic feedstock requires technical improvements to achieve economical production[1]. Recycle of the cellulase enzymes after hydrolysis of the substrate could improve the economics of the process. A major problem in cellulase recycle is the adsorption of the enzyme onto the substrate[2,3]. Vallander and Eriksson[2] found that enzyme recycle is feasible when the enzyme-residue complex is also recycled, however this procedure will result in increasing substrate levels due to lignin accumulation. Our previous paper[3] concluded that quantitative recovery of enzyme used in hydrolysis might depend on obtaining high levels of conversion. The optimum level of enzyme loading for hydrolysis using the enzymes derived from T. harzianum was determined to be 50 IU filter paper activity/g substrate[4]. For a commercial process, the substrate loading will have to be greater than 10% to keep process costs to a minimum. The present paper investigates enzyme recycle under process conditions of 10% substrate and 50 FPU/g enzyme loading.

Steam-pretreated aspenwood, following water-and-alkali extraction, is routinely used for both hydrolysis and enzyme production. Use of the substrate for enzyme production reduces the glucose yield obtained per tonne of wood. The amount of substrate required for enzyme production would be reduced if the residue remaining after hydrolysis could be recycled towards cellulase production.

MATERIALS AND METHODS

Cellulase preparation

The fungus Trichoderma harzianum E58 was taken from the Forintek Culture Collection. A spore inoculum was used to initiate growth in shake flasks containing Vogel's[5] medium and 1% glucose. After 3 days at 28°C a mycelium suspension was used to initiate growth in 500-mL Erlenmeyer flasks containing the cellulosic material in 150 mL Vogel's medium. After 4 days incubation at 28°C, the culture filtrates were harvested by filtration through a Whatman glass microfibre filter.

The cellulase preparation used for the hydrolysis of 10% substrate was produced in a 28-L New Brunswick fermentor with a working volume of 20 L. The culture filtrate was concentrated by ultrafiltration using a Pellicon Cassette System (Millipore), fitted with a polysulfone membrane with a molecular cut-off of 10,000 dalton, then freeze dried.

The purified exoglucanase, derived from T. harzianum E58, was kindly donated by Dr. L.U.L. Tan.

Assays

Total reducing sugars were determined colorimetrically using dinitrosalicylic acid reagent[6].

Enzymatic activity was determined by the method of Mandels et al[7]. One millilitre of a properly diluted culture filtrate was added to 1 mL 0.05M citrate buffer at pH 4.8. The substrates for the filter paper, endoglucanase, and β-glucosidase assays were a 1 x 6-cm strip of Whatman No. 1 filter paper, 10 mg carboxymethyl cellulose (CMC medium viscosity, Sigma C-4888) and 10 mg salicin, respectively. All three assays were performed at 50°C. The filter paper assay was incubated for 1 h, while the endoglucanase and β-glucosidase assays were incubated for 30 min. All three assays were terminated by adding 3 mL dinitrosalicylic acid reagent to each tube and boiling the tubes for 5 min. The tubes were cooled to room temperature and the absorbance read at 575 nm. One unit of enzyme activity was defined as 1 μmol glucose equivalents released per minute.

Sugar Analysis

The sugars produced during enzymatic hydrolysis were analyzed on a Varian HPLC system (Model 5000) interfaced with a Varian CDS 401 data system. Monitoring was performed using a Varian RI-3 refractive index detector. An HPX-87H column (Bio-Rad) was operated with a guard column (Cation H; Bio-Rad) using 0.01N sulphuric acid as the eluent. The flow rate was 0.6 mL/min, and the column temperature was 70°C.

Substrate preparation

Aspenwood chips were saturated with steam at 240°C for 80 s, then steam-exploded. A 5% slurry of the material was sequentially extracted with water (2x1h) and 0.4% NaOH (2x1h). This material, SEA-WAI, was washed thoroughly with water, until the washings were neutral. The chemical composition of SEA-WAI was 84.4% cellulose and 9.0% total apparent lignin.

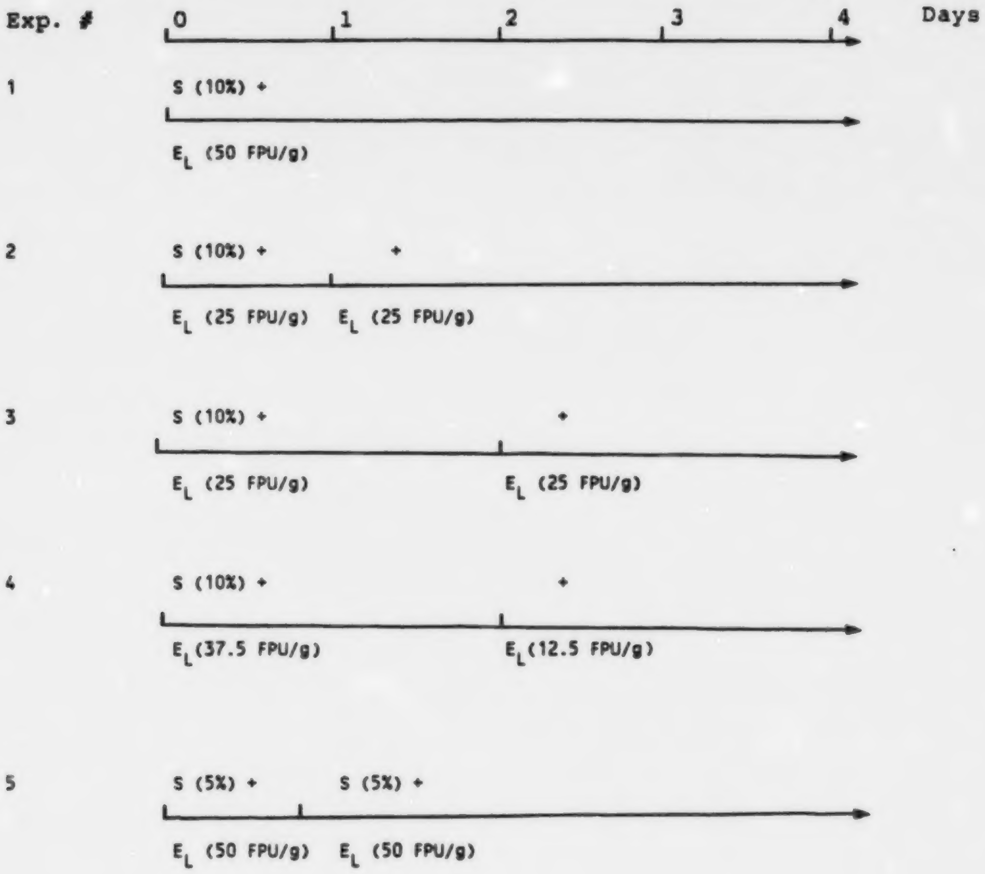
The hydrolysis residue, SEA-WAI, HR, was obtained by enzymatic hydrolysis of SEA-WAI. The unhydrolyzed residue was collected by centrifugation at 10,000 rpm for 30 min. Soluble sugars were removed by resuspension of the pellet in distilled water and recentrifugation.

RESULTS

For economical reasons it was desirable to hydrolyze the steam-treated, water-and-alkali-washed aspenwood at a 10% concentration (w/v). Steam treated wood has a low bulk density and agitation of a 10% concentration is difficult. Complete conversion at this level requires long residence times, thereby increasing the amount of enzyme inactivation. A way of overcoming these problems was to add the substrate and enzyme to the hydrolysis reactor in a fed-batch addition mode. Figure 1 shows various combinations of adding the

Figure 1.

Comparison of protocols for enzymatic hydrolysis of 10% SEA-WAI at an enzyme loading of 50 FPU/g substrate.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S (10%) +
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E_L (25 FPU/g)
E_L (25 FPU/g)
4
S (10%) +
+
E_L(37.5 FPU/g)
E_L(12.5 FPU/g)
5
S (5%) +
S (5%) +
E_L (50 FPU/g)
E_L (50 FPU/g)

S= Substrate, E_L= Enzyme Loading (expressed as FPU/g substrate present)

Table 1.

Hydrolysis of 10% SEA-WAI using protocols outlined in Figure 1.

Experiment No.	Hydrolysis Products[1] Glucose, g/L	 Cellobiose, g/L	Percent[2] Hydrolysis
1	83.1	7.4	96.9
2	81.6	7.6	95.6
3	80.2	7.8	94.3
4	83.1	8.2	97.8
5	85.1	8.5	100.3

[1] Sugar determinations were performed by HPLC.

[2] Glucan content of SEA-WAI was 84.4%.

substrate and enzymes for the hydrolysis of 10% SEA-WAI. High conversion levels were achieved for all the systems (Table 1), with the highest hydrolysis level obtained when a double fed-batch addition mode (i.e. two-step addition of enzymes and substrate) was used.

The culture filtrates from the various hydrolysis reactions were ultrafiltered using an ultrafiltration unit fitted with a 10 kd molecular weight cut-off membrane. The cellulase enzymes and other high molecular weight compounds were retained by the membrane, while the sugars and other low molecular weight compounds produced during hydrolysis were removed (Table 2). Recovery of endoglucanase and filter paper activity was low, while recovery of β-glucosidase activity was much higher. The low recovery of cellulase enzymes following extensive hydrolysis was disappointing, and hard to reconcile with the low level of glucan remaining in the residue. The original substrate contained 84.4% cellulose and 9.0% lignin, so after extensive hydrolysis a lignin-rich residue remains. Other researchers[8,9] have demonstrated the cellulase enzymes have a strong affinity for the lignin-carbohydrate complex. Adsorption of the cellulases was probably partially responsible for the low recovery values.

A major problem in adsorption studies is that the activity of the free enzymes is measured, and adsorption of the remaining enzyme is inferred. After 4 days hydrolysis a portion of the enzyme system may have been denatured, but our test would have reported these as adsorbed enzymes. The thermal stability of the enzymes may be such that little activity remained after 96 hours at 45°C, or alternately degradative products may be formed during hydrolysis which denatured the enzymes. These concerns were partially addressed by examining the extent of thermal inactivation during hydrolysis. Enzyme stability over a four day incubation period was studied. The enzyme system was buffered and agitated to duplicate the hydrolysis conditions, but in the absence of substrate. The endoglucanase component appeared to be the most thermal-labile, losing 20% of its activity over a 4 day period (Figure 2). The preparation lost 10-15% of the filter paper and β-glucosidase activities, respectively, after 4 days. In order to evaluate the effect of thermal

Table 2.

Recovery of cellulase enzymes following hydrolysis of 10% SEA-WAI.

Experiment No.	Percent[1] Hydrolysis	Enzyme Activities (% of original)[2] Endoglucanase	β-glucosidase	Filter Paper
3	94.3	8.6	39.9	10.8
2	95.6	11.5	41.6	16.1
1	96.9	15.2	43.0	22.9
4	97.8	12.0	46.0	16.9
5	100.3	12.4	40.6	17.5

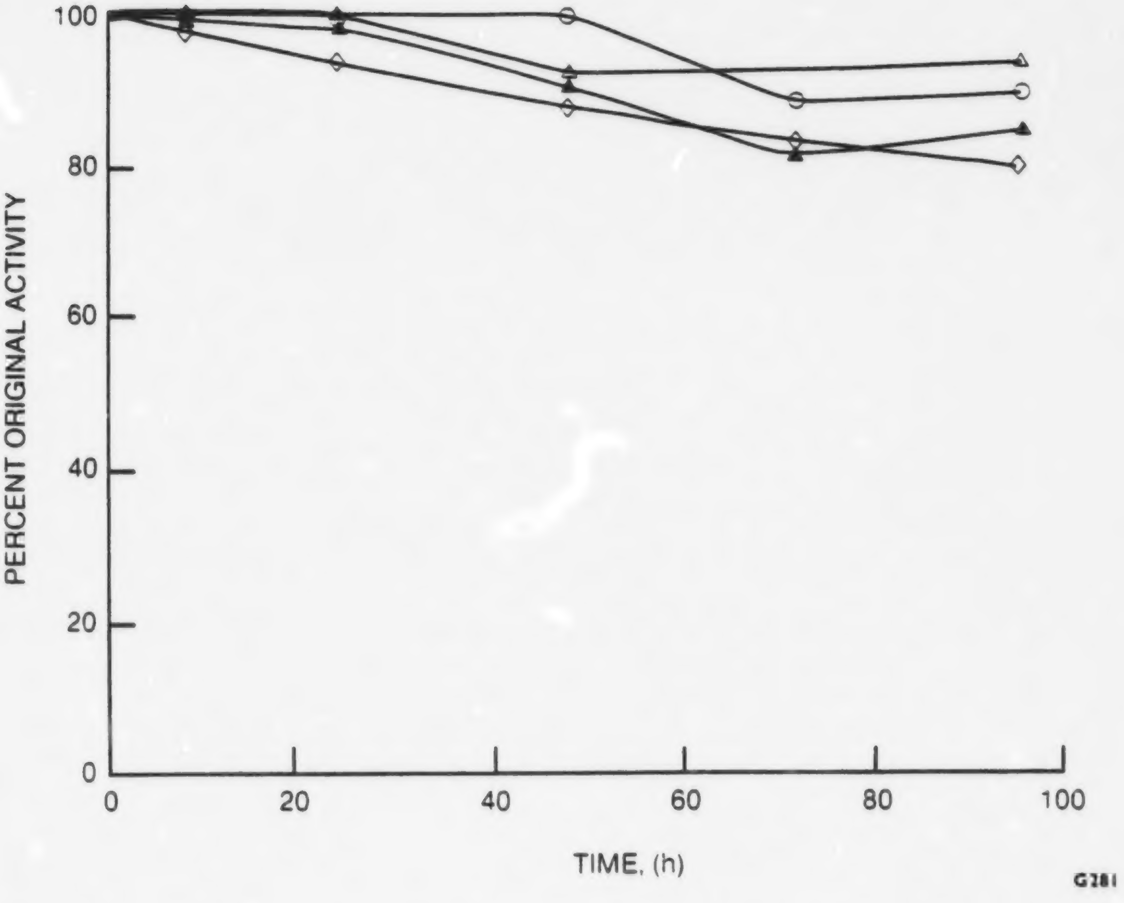
Enzymes were recovered from the culture filtrates as described in Materials and Methods.

[1] Hydrolysis was performed as described in Figure 1.

[2] Original enzyme activity was endoglucanase, 86.6 IU/mL; Filter paper activity, 5.0 FPU/mL; and β-glucosidase, 5.2 IU/mL.

Figure 2.

Thermal stability of the endoglucanase (◊), β-glucosidase (▲), and filter paper (○) activities of T. harzianum E58 cellulases and purified exoglucanase (△) activity after incubation at 45°C, pH 4.8.

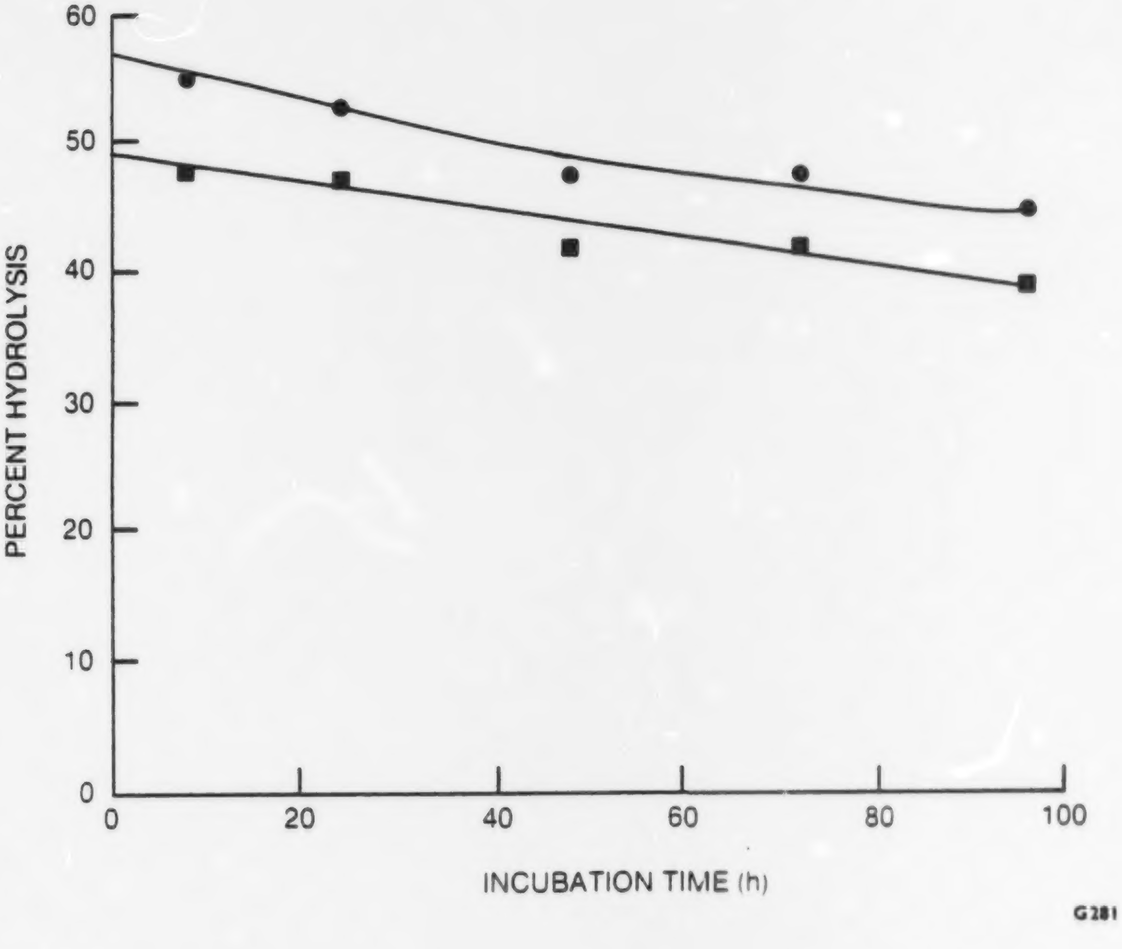


Enzyme activity (IU/mg protein) profile for the cellulase preparation was:
Endoglucanase, 13.30
β-glucosidase, 0.45
Filter paper activity, 0.67

Protein concentration of the incubated cellulases was 1.12 mg/mL.
Protein concentration of the incubated purified exoglucanase was 1.04 mg/mL.

Figure 3.

Effect of thermal inactivation on the hydrolytic efficiency of the preincubated cellulases to degrade a 2% (w/v) concentration of Solka Floc BW300 to reducing sugars (●) and glucose (■).



Enzyme fractions were collected at various times during incubation at 45°C (Figure 2). The enzyme solutions were filter-sterilized and added, without dilution, to the hydrolysis systems. After 24 h incubation at 45°C the amount of sugars released by the various systems were compared.

Initial enzyme loading, at t=0 h, was 30 FPU/g substrate.

Table 3.

Hydrolysis[1] of 10% SEA-WAI using fresh cellulase enzymes supplemented with various recovered cellulase enzymes.

Source of Recovered Enzymes[2] Exp. No.	Enzyme Loading, IU/g			Hydrolysis Products		Percentage Hydrolysis
	Endoglucanase	β-glucosidase	Filter Paper Activity	Glucose g/L	Cellobiose g/L	
3	419.8	38.1	23.1	59.0	6.4	70.1
2	435.7	38.7	24.8	61.2	6.5	72.6
1	456.6	39.1	27.0	63.3	6.5	74.8
4	438.7	40.2	25.1	63.2	6.8	75.0
5	438.8	38.0	25.1	64.2	6.8	76.1
Fresh-only Control	371.9	24.8	19.6	61.9	9.1	76.2

[1] Hydrolyses were performed as described in Figure 1.

[2] Recovered enzymes were ultrafiltered as described in Materials and Methods. The enzymes were added to fresh cellulase enzymes to give the above enzyme loadings.

Table 4.

Batch hydrolysis[1] of 10% SEA-WAI at an enzyme loading of 50 FPU/g substrate.

Incubation Time, h	Hydrolysis Products Glucose g/L	Cellobiose g/L	% Residual Cellulose[2]
4	20.4	4.4	61.9
8	28.6	4.5	54.4
24	53.4	4.9	31.7
48	68.7	4.3	18.5
96	79.4	3.8	9.3
168	87.6	3.9	1.9

[1] Hydrolysis was performed at 45°C with cellulases derived from *T. harzianum* E58.

[2] Original cellulose content was 84.4%.

inactivation on the hydrolytic efficiency of the cellulases, the ability of these cellulases to hydrolyze cellulose was tested. Enzyme samples were recovered at various times during incubation at 45°C, then incubated with fresh crystalline cellulose for 24 hours at 45°C (Figure 3). Enzymes that had been preincubated for 4 days at 45°C had lost only 20% of their ability to degrade Solka Floc BW300 to glucose. The results indicated enzyme stability is a contributing factor to enzyme loss during hydrolysis.

The glucose-to-cellobiose ratio was approximately 9:1 in all the hydrolysis modes examined (Table 1) indicating there was insufficient β-glucosidase in the enzyme preparation. Conversion to glucose may be enhanced by supplementing the systems with β-glucosidase. As the β-glucosidase recovery was fairly high it was worthwhile examining the recycled enzymes as a source of supplemental β-glucosidase.

The recovered enzymes were combined with fresh enzyme and used to hydrolyze 10% SEA-WAI (Table 3). The activities of the different systems were assayed and in all cases the supplemented systems had greater enzyme activities than the control. After hydrolysis, the systems supplemented with the recovered enzymes had less cellobiose built-up than the control, due to the additional β-glucosidase. There was not, however, an increase in the extent of hydrolysis as was expected with the higher cellulase loading. In some cases the percentage hydrolysis decreased with the addition of recovered enzymes.

The data suggested that substances present in the recovered cellulase fraction may be inhibitory to enzyme action. It is known that oxidized phenolic compounds can attach themselves to proteins and react covalently to inactivate many enzymes[10]. Phenolic compounds, derived from the lignin fraction, may be bound to the cellulases and would be retained with the enzyme fraction during ultrafiltration. The source of the inhibition was further investigated by examining the high molecular weight fraction after 4 days hydrolysis of 10% SEA-WAI (Table 4). After ultrafiltration through a 10 kd membrane, the high molecular weight fraction was boiled to denature the cellulases. Half of this mixture was filtered to remove the precipitated proteins, while the other half was sonicated to obtain a suspension of the inactivated proteins. The two systems were combined with fresh cellulases and applied to hydrolysis of a 10% concentration of Solka Floc BW300.

The high molecular weight fractions had no significant effect on the long term hydrolysis in comparison to the control. Apparently, there are no inhibitors present in the high molecular weight fraction. Klyosov has demonstrated a correlation between the tightness of cellulase adsorption and the efficiency of their catalytic effect on crystalline cellulose[11]. Similarily, Beldman _et al_[12] divided _Trichoderma viride_ endoglucanases into two groups; one that adsorbed strongly to crystalline cellulose and one that did not. The enzymes most capable of attacking crystalline cellulose may remain bound to the residue, while the weakly adsorbed enzymes remain in solution and are available for recycling. The addition of these poorer quality enzymes to fresh substrate may have little or no impact on the efficiency of hydrolysis, and may interfere in hydrolysis by competing for adsorption sites with the crystalline-degrading cellulases.

The first stage in enzyme production is preparation of the seed inoculum, a step which requires a readily-consumed carbon source. For the wood-to-glucose process to be commercially feasible the glucose produced in-house must be cheaper than purchased glucose. The glucose produced in-house would be an ideal carbon source for inoculum production, however, inhibitors present in the hydrolysis stream may interfere with fungal growth. Growth of _T. harzianum_ E58 on the sugar syrups produced during batch hydrolysis of 10% SEA-WAI was investigated. The hydrolyzates were collected at various times during hydrolysis and diluted to obtain a 1% sugar concentration in the media. After 3 days incubation all the available sugars had been consumed for all the systems (Table 5). Biomass yields were comparable for all the systems, indicating that the presence of inhibitors was not a problem for fungal growth at this sugar concentration.

The production of the enzymes in a wood-to-glucose process is a costly component. The best substrate for high enzyme production in _T. harzianum_ E58 appears to be a cellulosic material[13] and the most feasible candidate would be

Table 5.

Use of cellulose hydrolyzates for production of *T. harzianum* E58 biomass.

Time, h	% Hydrolysis	C/G Ratio	Biomass Yield g/g sugars
4	38.1	0.22	0.53
8	45.6	0.16	0.53
24	68.3	0.09	0.52
48	81.5	0.06	0.54
96	90.7	0.05	0.58
168	98.1	0.04	0.56
Control		0.00	0.58

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[1] *T. harzianum* E58 was grown on 1% sugar for 4 days, 28°C.

[2] Hydrolysis was performed on 10% SEA-WAI at an enzyme loading of 50 FPU/g substrate, 45°C (Table 4). The ratio of cellobiose (C) to glucose (G) in the hydrolyzates is given above.

the incoming feedstock. This approach, however, decreases the potential glucose from the feedstock as some of the feedstock must be diverted to enzyme production. The long hydrolysis time and high enzyme loading required for complete hydrolysis makes it attractive to look at a hydrolysis scheme where the target would be 80-90% hydrolysis of the cellulose component. In such a scheme the accumulation of lignocellulosic residue from the hydrolysis reactor would be high and a waste treatment plant would be required to dispose of the material. An alternative plan is to use this residue as the cellulose source for enzyme production. Hydrolysis of cellulose generally results in the formation of a highly indigestible form of cellulose[14]. The production of cellulase enzymes is partially controlled by the accessibility of the cellulose to the cellulase enzyme[13], with the more recalcitrant celluloses inducing greater enzyme production. Early work demonstrated the feasibility of this approach with Solka Floc, a pure cellulose substrate[3]. Other researchers have obtained promising results using the residue remaining after hydrolysis of wheat straw[15]. A major concern is that the high amount of lignin present in the residue may have a negative impact on the use of this cellulose substrate. Vohra and co-workers[16] noted lignin and its components had a negative effect on enzyme production in _T_. _reesei_.

Our first attempts at using the residue from the hydrolysis of steamed aspenwood for cellulase production were unsuccessful (Table 6). Prior to using the residue for growth the residue was dried, which may have had a detrimental effect on the structural properties of the substrate. When the residue was kept wet, and combined with fresh steam-treated wood, high levels of cellulase enzymes were produced (Table 7). As hydrolysis proceeded the cellulosse content of the substrate decreased, while the lignin concentration increased. The cellulase production per gram of substrate, however, was found to be inversely proportional to the cellulose content (Table 8). The hydrolytic activity of the produced filtrates against 2% SEA-WAI was tested (Table 9). Glucose yields were high, while cellobiose build-up was low (<2% of the cellulose content), indicating that inhibition of either the cellulases or β-glucosidase did not appear to be a problem.

Table 6.

Enzyme production by *Trichoderma harzianum* E58 grown for 4 days on SEA-WI, HR[1].

	Enzyme Activity, IU/mL		
Concentration %	Endoglucosidase	β-glucosidase	Filter paper Activity
1	3.0	0.13	0.16
2	4.1	0.27	0.24

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[1] SEA-WI, HR is the residue remaining after a combined enzymatic hydrolysis and fermentation of 5% SEA-WI.

Table 7.

Cellulase enzyme production by *T. harzianum* E58[1] grown on SEA-WAI and SEA-WAI, HR[2].

Growth Substrate	Ratio	Enzyme Activities, IU/mL Endoglucanase	 β-glucosidase	 Filter Paper Activity
SEA-WAI	100	10.6	0.53	0.61
SEA-WAI: SEA-WAI, HR^{24h}	75:25	10.4	0.56	0.55
	50:50	10.6	0.58	0.72
SEA-WAI: SEA-WAI, HR^{48h}	75:25	10.4	0.58	0.56
	50:50	11.7	0.65	0.85
SEA-WAI: SEA-WAI, HR^{96h}	75:25	10.9	0.61	0.64
	50:50	12.4	0.70	0.85
SEA-WAI: SEA-WAI, HR^{168h}	75:25	9.8	0.60	0.67
	50:50	11.5	0.74	0.74

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[1] *T. harzianum* E58 was grown on 1% substrate for 4 days at 28°C.

[2] SEA-WAI, HR$^{t\ hr}$ is the hydrolysis residue remaining after hydrolysis of 10% SEA-WAI for various lengths of time, as described in Table 4.

Table 8.

Cellulase enzyme yield from growth of *T. harzianum* E58[1] grown on SEA-WAI and SEA-WAI, HR[2].

Growth Substrate	Ratio	Cellulose Content %	Enzyme Yield, IU/g cellulose		
			Endoglucanase	β-glucosidase	Filter Paper Activity
SEA-WAI	100	84	1262	63.1	72.6
SEA-WAI: SEA-WAI, HR^{24h}	75:25	71	1465	78.9	77.5
	50:50	58	1828	100.0	124.1
SEA-WAI: SEA-WAI, HR^{48h}	75:25	68	1529	85.3	82.4
	50:50	51	2294	127.5	166.7
SEA-WAI: SEA-WAI, HR^{96h}	75:25	66	1652	92.4	97.0
	50:50	47	2638	148.9	180.8
SEA-WAI: SEA-WAI, HR^{168h}	75:25	64	1531	93.8	104.7
	50:50	43	2674	172.1	172.1

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[1] *T. harzianum* E58 was grown on 1% substrate for 4 days at 28°C.

[2] SEA-WAI, HR$^{t\ hr}$ is the hydrolysis residue remaining after hydrolysis of 10% SEA-WAI for various lengths of time, as described in Table 4.

Table 9.

Hydrolytic potential of cellulase enzymes produced by *T. harzianum* E58[1] grown on SEA-WAI and SEA-WAI, HR[2].

Growth Substrate	Ratio	Enzyme Loading IU/g	Percentage Hydrolysis of 2% SEA-WI[3] to Glucose		
			Day 1	Day 2	Day 3
SEA-WAI	100	15.3	49.7	64.8	84.2
SEA-WAI: SEA-WAI, HR^{24h}	75:25	13.8	51.8	67.7	85.7
	50:50	18.0	54.7	70.6	87.8
SEA-WAI: SEA-WAI, HR^{48h}	75:25	14.0	46.1	56.9	75.6
	50:50	21.3	60.5	74.9	91.4
SEA-WAI: SEA-WAI, HR^{96h}	75:25	16.0	54.7	69.1	87.1
	50:50	21.3	63.4	77.8	92.9
SEA-WAI: SEA-WAI, HR^{168h}	75:25	16.8	51.1	64.1	84.2
	50:50	18.5	59.8	73.4	90.0

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[1] *T. harzianum* was grown on 1% substrate for 4 days.

[2] SEA-WAI, $HR^{t\ hr}$ is the hydrolysis residue remaining after hydrolysis of 10% SEA-WAI for various lengths of time, as described in Table 4.

[3] Glucan content of SEA-WI was 62.5%.

At higher substrate concentrations these residues may prove inhibitory to enzyme production. A 1 and 2% substrate concentration of the lignin residues, obtained after 72.2 and 87.6% hydrolysis of the substrate, was used for enzyme production (Table 10). Higher concentrations of the residue had a detrimental effect on enzyme production, the effect more marked with the more hydrolyzed fraction. In comparison, the fresh substrate, SEA-WAI, demonstrated only a slight decrease in enzyme production at a 2% substrate concentration.

DISCUSSION:

The low recovery of cellulase enzymes from the hydrolysis of 10% SEA-WI could be attributed to three factors. The adsorption of the enzymes onto the residue probably played the major role, although thermal stability of the enzymes and inactivation due to presence of inhibitors also contributed to the low recoveries. A 20% loss of enzymes can be attributed to thermal stability. The relative importance of adsorption and inhibition is difficult to ascertain. Previous data[3] indicates that enzymatically treated steamed aspenwood with a cellulose content of 3-10% can adsorb 33% endoglucanase, 43.6% filter paper activity and 15% β-glucosidase. Enzyme recovery after hydrolysis was 13% endoglucanase, 16% filter paper and 51% β-glucosidase. In this hydrolysis 54% of the endoglucanase, 40% filter paper activity and 34% β-glucosidase loss must be attributed to other factors such as thermal stability (20%) and inactivation due to inhibitory substances, such as lignin[8]. As the concentration of the steam treated material increases the loss of enzyme activity due to presence of inhibitory substances may increase.

The presence of lignin-derived inhibitors and the adsorptive properties of the lignin/cellulose residue posed serious limitations to the technical feasibility of enzyme recycle following hydrolysis of steamed wood. The lignin-rich residue strongly adsorbed the cellulase enzymes, making them unavailable for recycle. More extensive delignification of the substrate may be necessary for successful enzyme recycle.

Table 10.

Cellulase enzyme production by *T. harzianum* E58 grown on SEA-WAI or SEA-WAI, HR.

Growth Substrate	Cellulose Content %	Enzyme Activities, IU/mL			Percentage Hydrolysis of 2% SEA-WAI to Glucose		
		Endoglucanase	β-glucosidase	Filter Paper Activity	Day 1	Day 2	Day 3
1% SEA-WAI	84	7.8	0.63	0.59	49	62	80
2% SEA-WAI		7.6	0.64	0.49	47	61	80
1% SEA-WAI, HR^{96h}	28	10.5	0.79	0.75	56	66	85
2% SEA-WAI, HR^{96h}		7.2	0.74	0.60	50	63	82
1% SEA-WAI, HR^{168h}	13	6.2	0.70	0.64	52	65	84
2% SEA-WAI, HR^{168h}		4.9	0.63	0.44	43	54	73
0.5% SEA-WAI + 0.5% SEA-WAI, HR^{96h}	56	9.4	0.74	0.70	55	69	87
0.5% SEA-WAI + 0.5% SEA-WAI, HR^{168h}	48	11.2	0.75	0.75	59	70	87

The hydrolyzate syrups were found to be suitable for biomass production. These syrups had not been processed, indicating the inhibitors present in the hydrolyzate did not affect fungal growth.

The residual material from enzyme hydrolysis was found to be a good carbon source for enzyme production. This result supports earlier work, where it was shown that the more recalcitrant substrates promote high cellulase production[3]. Studies have shown that this process modification can increase the potential glucose from the incoming feedstock by 20%[4]. This material has only been used at low substrate concentration and problems with inhibitory materials may surface when higher substrate concentrations are used but this appeared to be dependent on the cellulose composition of the substrate.

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V. FERMENTATION OF HEMICELLULOSE

A SIMPLE METHOD FOR XYLANASE PREPARATION USED FOR THE HYDROLYSIS AND FERMENTATION OF HEMICELLULOSE TO BUTANEDIOL
E.K.C. Yu, L. Deschatelets, L.U.L. Tan and J.N. Saddler, 1985
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A simple method for xylanase preparation used for the hydrolysis and fermentation of hemicellulose to butanediol

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SUMMARY

When culture filtrates of Trichoderma harzianum E58 were concentrated by passage through an ultrafiltration membrane with a molecular weight exclusion limit of 10,000 Daltons, 80% of the original xylanase activity was recovered in the ultrafiltrates. Culture filtrates and ultrafiltrates which were concentrated by rotary evaporation contained inhibitors which restricted the fermentation of the hemicellulose-derived sugars to 2,3-butanediol. A simple solvent-exchange treatment of the ultrafiltrates could effectively concentrate the xylanases as well as remove the fermentation inhibitors.

INTRODUCTION

Hemicelluloses constitute 20-35% by weight of wood and agricultural residues and can serve as an abundant and inexpensive source of fermentable carbohydrates (Flickinger, 1980). The efficient utilization of the hemicellulose fraction would greatly enhance the economics of many of the bioconversion processes which currently only emphasized the conversion of the cellulose component to fuels and chemicals. We have recently used a combined enzymatic hydrolysis and fermentation (CHF) approach to directly convert hemicellulose to butanediol (Yu et al, 1984a; 1984b). Enzymatic hydrolysis was found to be the rate-limiting step in the CHF process, suggesting that an increase in enzyme concentration would enhance the efficiency of the overall process. We therefore studied various ways of concentrating the active xylanase enzymes present in the culture filtrates of Trichoderma harzianum E58. The work described in this paper shows that a simple solvent-exchange treatment could be used to concentrate the xylanases present in the ultrafiltrates and, at the same time, remove inhibitors present in the enzyme preparations which restricted the fermentation of the hemicellulose-derived sugars to butanediol.

MATERIALS AND METHODS

Klebsiella pneumoniae ATCC 8724 was grown in a chemically defined medium (Kp 2) under finite air conditions (Yu and Saddler, 1982a; 1982b). Xylanase enzyme preparations were obtained from the culture filtrates of Trichoderma harzianum E58 grown on 1% (w/v) solka floc in Vogel's medium (Saddler, 1982). Culture filtrates were concentrated by rotary evaporation or by ultrafiltration through Pellicon membrane with a molecular weight exclusion limit of 10,000 Daltons (Pellicon Cassette System, Millipore Ltd., Mississauga, Ontario, Canada). Assays for cellulolytic and xylanolytic enzyme activities were described previously (Yu et al, 1984b).

Ultrafiltrates (Pellicon filtrates) were concentrated by solvent exchange treatment. The Pellicon filtrate containing 0.7 g total protein was diluted with three volumes of 10 mM ammonium acetate buffer (pH 5.0) and applied onto a 5 cm by 21 cm CM-Sepharose column (Pharmacia Fine Chemicals) equilibrated with the dilution buffer. The column was washed with 4 litres of the starting buffer. Elution was effected with 2.5 litres of 50 mM sodium phosphate buffer (pH 8.0). The xylanase activity peak was pooled and used for the CHF process.

Hemicellulose (aspenwood xylan) used in the present study was prepared by alkali extraction of extractive-free aspenwood sawdust (Jones et al, 1961; Koshijima et al, 1965).

Hemicellulose conversion to butanediol was carried out using the combined enzymatic hydrolysis and fermentation approach (Yu et al, 1984a). Culture supernatant fluids were analyzed for volatile fermentation products by gas chromatography (Yu and Saddler, 1982b). Reducing sugars were assayed with dinitrosalicylic reagent (Miller, 1959). Xylose and xylobiose were assayed by high performance liquid chromatography (Wentz et al, 1982).

RESULTS

Culture filtrates of Trichoderma harzianum E58 exhibited high cellulase and xylanase enzyme activities (Table 1). Rotary evaporation of the culture filtrates resulted in the effective concentration of all of the corresponding enzymes. Ultrafiltration of the culture filtrates by passage through a Pellicon membrane significantly concentrated the cellulases and xylobiase enzymes in the Pellicon retentates, however 80% of the original xylanase activities was found in the Pellicon filtrate. These low molecular weight xylanases could be concentrated either by rotary evaporation or by solvent-exchange and highly active homogeneous xylanase fractions were obtained by both these methods.

We next investigated the mode of hydrolysis of aspenwood xylan, using these various enzyme preparations (Table 2). Although the concentrated culture filtrates were all adjusted to the same level of xylanase activity, the Pellicon retentates appeared to be the most efficient at hydrolyzing xylan to reducing sugars with a large proportion of the sugars detected as xylose. The Pellicon filtrates appears to be less hydrolytic. Significantly more xylobiose was detected in the hydrolyzates resulting in lower overall xylose to xylobiose ratios. This higher xylobiose content was presumably because the majority of the xylobiase activity was located in the retentate resulting in an ultrafiltrate which was deficient in this enzyme.

Since Klebsiella pneumoniae can utilize both xylose and xylobiose (Yu et al, 1984b), the relative ratio of xylose to xylobiose obtained after hydrolysis was not expected to significantly affect the final butanediol yields obtained when a CHF process was used. When the four different culture filtrates were used as the enzyme source in a CHF process, using previously established conditions (Yu et al, 1984a), lower butanediol yields were obtained with both the rotary evaporated Pellicon filtrate and the rotary evaporated culture filtrates (Fig. 1). This seemed to indicate that there were fungal metabolites or fungal medium ingredients in the concentrated enzyme preparations which inhibited fermentation of the liberated sugars. The effect of the fungal (Vogel's) medium ingredients on butanediol fermentation was therefore examined (Table 3). By systematically supplementing the butanediol fermentation medium with various components of the Vogel's medium, it was shown that the inhibitory effect observed was due to the salts present in the Vogel's medium. No single salt component in the Vogel's medium was found to be solely responsible for the observed inhibition, suggesting that the inhibition was a result of the synergistic action of the various salts. We have tried to modify the salt composition of the Vogel's medium so that the resulting culture filtrates would be more suitable for hydrolysis and fermentation studies. However, all modifications investigated to date resulted in a significant decrease in the xylanase enzyme production by the fungus.

We next turned our attention to practical methods which could effectively concentrate the xylanase enzymes while at the same time remove the fermentation inhibitors. This was achieved by treating the Pellicon filtrates by a simple solvent-exchange procedure. The final butanediol levels achieved in the CHF of xylan using the solvent-exchanged

Fig. 1 Butanediol production by Klebsiella pneumoniae grown in a CHF process on 10% (w/v) xylan using various T. harzianum E58 culture filtrate fractions

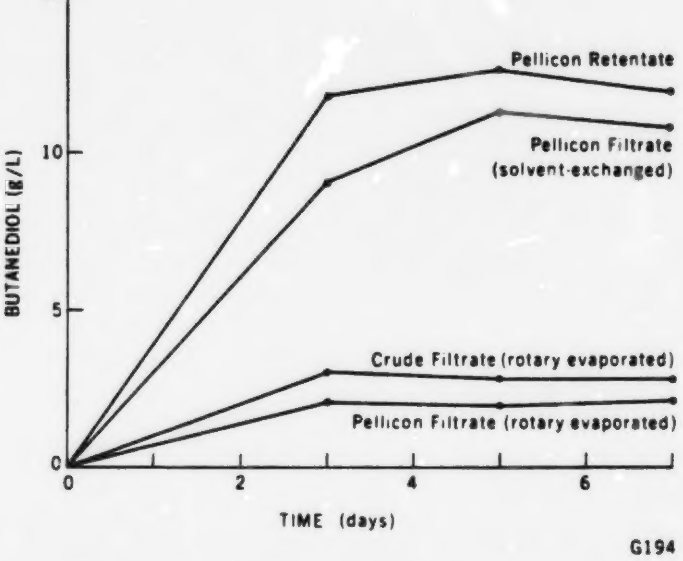
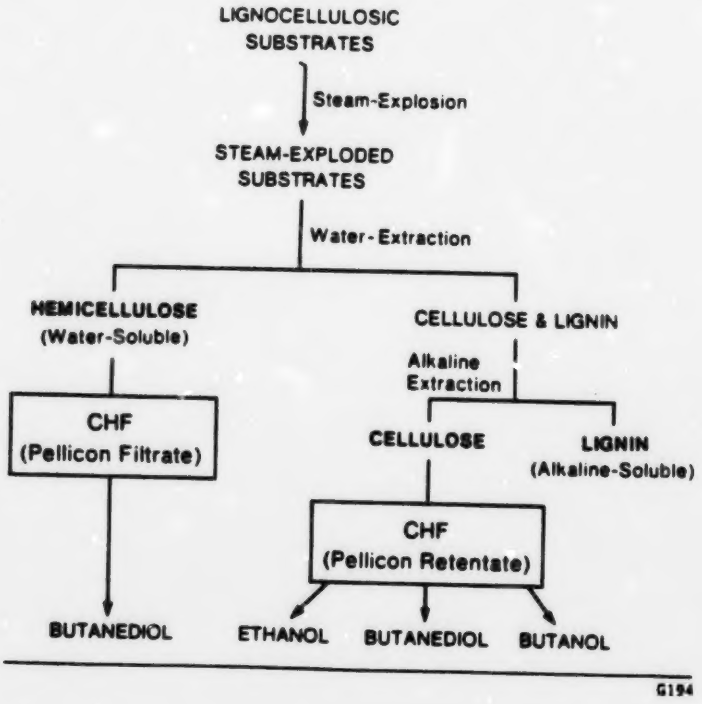


Fig. 2 Process schemes for the fractionation and subsequent utilization of lignocellulosic substrates


LIGNOCELLULOSIC SUBSTRATES
Steam-Explosion
STEAM-EXPLODED SUBSTRATES
Water-Extraction
HEMICELLULOSE (Water-Soluble)
CELLULOSE & LIGNIN
Alkaline Extraction
CHF (Pellicon Filtrate)
CELLULOSE
LIGNIN (Alkaline-Soluble)
CHF (Pellicon Retentate)
BUTANEDIOL
ETHANOL
BUTANEDIOL
BUTANOL
G194

xylanase preparation were comparable to those obtained by a corresponding CHF process using the Pellicon retentates as the enzyme source (Fig. 1). Under the established optimal conditions, total solvent yields of up to 20.5 g/L (butanediol, 14.3 g/L; acetoin, 0.8 g/L; ethanol, 5.1; acetic acid, 0.3) could be obtained directly from xylan (10%, w/v) within three days of the CHF process (data not shown). The overall bioconversion yields were 0.30 g of butanediol and 0.41 g of total solvents produced per g of sugar utilized. The results clearly demonstrated the potential of using this method for recovering the xylanase enzymes in the Pellicon filtrates to achieve effective hydrolysis and fermentation of hemicellulose to butanediol.

DISCUSSION

The full utilization of the lignin, cellulose, and hemicellulose components of lignocellulosic substrates might enable a bioconversion process to compete economically with petrochemical processes. This would require the fractionation of the biomass residues into their respective components prior to the conversion of each component to useful products. By pretreating lignocellulosic substrates by steam explosion (Fig. 2) we have shown that biomass substrates can be effectively fractionated into a hemicellulose-rich fraction by a simple water-extraction of the steamed substrates, a lignin-rich component, by a subsequent alkaline extraction of the water-extracted residues, leaving a cellulose-rich residue. Since both the cellulose and hemicellulose are almost exclusively in oligosaccharide forms (Saddler et al, 1982; Yu et al, 1984a), they have to be hydrolyzed to their component sugars prior to fermentation. Recently we have simplified the process by using the combined hydrolysis and fermentation (CHF) approach (Fig. 2). To maximize the rates and efficiencies of enzymatic hydrolysis, it would be desirable to have the cellulase and xylanase enzymes present in a concentrated form (Yu et al, 1984a, 1984b). Ultrafiltration is a simple and effective way of concentrating the cellulase, and to a lesser extent, the xylanase enzymes. This method has the additional advantage of being easily scaled-up for use in any future commercial operations.

Culture filtrates concentrated by ultrafiltration provide an excellent enzyme source for subsequent hydrolysis and fermentation of both the cellulose and hemicellulose rich fractions. However, as the ultrafiltrate contains highly active xylanases it would probably be advantageous if this was not considered a waste stream but rather as the source of enzymes for hydrolysis of the hemicellulose fraction. The simple method of solvent-exchange developed in this study resulted in the effective concentration of the xylanase enzymes as well as the removal of the fermentation inhibitors, enabling efficient hydrolysis and fermentation of the hemicellulose substrates to butanediol. Moreover, the CM-Sepharose used in the solvent-exchange procedure could be readily regenerated for repeated use and could easily be scaled-up for larger scale operations. We intend to carry out further studies on the CHF of hemicelluloses at high substrate concentrations using a fed-batch approach to examine the possibility of using lower enzyme levels without loss of overall bioconversion efficiences.

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Table 1. The enzyme profile of *Trichoderma harzianum* E58 culture filtrates after concentration by various methods

Culture filtrate fraction[a]	Enzyme Activities (IU/mL)				
	Endoglucanase	β-glucosidase	Filter Paper	Xylanase	Xylobiase
CF	14.0	0.6	0.7	277.1	<0.1
CF conc.	78.0	5.5	5.9	2442.9	1.5
PR	659.3	31.3	31.0	3117.9	24.8
PF	0.3	<0.1	<0.1	149.9	<0.1
PF conc.	4.8	<0.1	0.3	1611.9	<0.1
PF (SE)	< 0.1	<0.1	<0.1	1253.9	<0.1

[a]
CF, crude culture filtrate
CF conc, CF concentrated (10x, v/v) by rotary evaporation
PR, Pellicon retentate (concentrated 65x, v/v)
PF, Pellicon filtrate
PF, conc, PF concentrated (10x, v/v) by rotary evaporation
PF (SE), PF treated by solvent-exchange

Table 2. Analysis of sugars released from aspenwood xylan after hydrolysis by various enzyme preparations[a]

Xylan Conc.	Culture filtrate fraction[b]	Reducing Sugars (g/L)	Xylose (X) (g/L)	Xylobiose (X2) (g/L)	X+X2 (g/L)	X/X2 Ratio
50 g/L	CF conc	29.6	12.2	4.4	16.6	2.8
	PR	38.0	18.6	3.4	22.0	5.5
	PF conc	25.2	7.6	9.0	16.6	0.8
	PF (SE)	25.4	9.8	6.2	16.0	1.6
100 g/L	CF conc	64.0	40.6	8.0	48.6	5.1
	PR	79.6	49.8	2.4	52.2	7.8
	PF conc	54.0	25.4	14.2	39.6	1.8
	PF (SE)	53.6	39.4	11.2	50.6	3.5

[a] Enzymatic hydrolysis was carried out in fermentation medium and incubated at 30°C and pH 6.5 for 2 days to serve as control for the corresponding CHF process. Enzyme levels were all adjusted to 5 IU of xylanase activity per mg of substrate

[b] See Table 1 for explanations on enzyme preparations

Table 3. The effect of Vogel's medium ingredients on butanediol production by *K. pneumoniae*[a]

Medium and Modification	% inhibition of Butanediol production[b]
Kp2 (control)	0.0
Vogel's medium[c]	96.2
Kp2 + culture filtrate	78.0
Kp2 + Vogel's medium	82.2
Kp2 + Vogel's salts	79.6
Kp2 + Vogel's vitamins	8.3
Kp2 + Vogel's trace elements	5.7
Kp2 + Vogel's bactepeptone	1.3
Kp + Vogel's Tween 80	3.3
Kp2 + Vogel's $CaCl_2$	12.9
Kp2 + Vogel's KH_2PO_4	9.6
Kp2 + Vogel's $MgSO_4$	0.0
Kp2 + Vogel's citrate	0.0
Kp2 + Vogel's NH_4NO_3	23.3

[a] Cultures were grown for 3 days on D-xylose (5%, w/v) under finite air conditions at 30°C, pH 6.5, and with shaking (150 rpm). Medium ingredients added were adjusted to final concentrations equivalent to the Vogel's medium composition.

[b] % inhibition was calculated as the inverse of the amount of butanediol produced in comparison with the control.

[c] Composition of Vogel's medium: salts (sodium citrate.$2H_2O$, 2.5 g/L; KH_2PO_4, 5.0 g/L; NH_4NO_3, 2.0 g/L; $MgSO_4.7H_2O$, 0.2 g/L; $CaCl_2.2H_2O$, 0.1 g/L); vitamins (biotin, 0.5 ug/L ; myoinositol, 2.0 mg/L; calcium panthothenate, 0.2 mg/L; pyrodoxine-HCl, 0.2 mg/L; thymine-HCl, 0.2 mg/L); trace elements (citric acid.H_2O, 5.0 mg/L; $ZnSO_4.7H_2O$, 5.0 mg/L; Fe $(NH_4)_2(SO_4)_2.5H_2O$, 1.0 mg/L; $CuSO_4.5H_2O$, 0.25 mg/L; $MnSO_4,2H_2O$, 0.05 mg/L; H_3BO_3, 0.05 mg/L; $Na_2Mo_2O_4.2H_2O$, 0.05 mg/L); Bactopeptone, 1.0 g/L; Tween 80, 2.0 mL; carbon source, 10 g/L.

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Running Title: Steam-pretreated aspenwood hemicellulose fermentation

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SUMMARY

Enzymatic hydrolysates of hemicellulose from steam-pretreated aspenwood were more fermentable than the acid hydrolysate after rotoevaporation or ethyl acetate extraction treatments to remove acetic acid and sugar- and lignin-degradation products prior to fermentation by Pichia stipitis CBS 5776. Total xylose and xylobiose utilization from 5.0% (w/v) ethyl acetate extracted enzymatic hydrolysate was observed with a $Y_{p/s}$ of 0.47 g ethanol/g total available substrate and $Q_{p\ Max}$ of 0.20 g $L^{-1}h^{-1}$ in 72 h batch fermentation.

INTRODUCTION

For the economic production of ethanol from wood hydrolysates it is essential that the hemicellulosic sugars, in addition to the hydrolyzed cellulose, be efficiently fermented. Separation of both hemicellulose and lignin from the cellulose following steam-pretreatment of aspenwood has been shown to benefit enzymatic cellulose hydrolysis (Schwald et al. 1988). In addition, the presence of lignin in steam-pretreated aspenwood has been shown to reduce subsequent enzymatic cellulose hydrolysis efficiency by adsorbing cellulases (Sutcliffe and Saddler 1986). Consequently, the separation of steam-pretreated wood into its three major fractions, cellulose, hemicellulose and lignin, prior to enzymatic hydrolysis and fermentation is necessary to improve the utilization efficiency of the total pretreated wood.

When aspenwood hemicellulose is obtained following a non-acid-catalyzed steam-pretreatment of 80 s at 240°C, it is primarily in the form of water-soluble pentosan (Brownell and Saddler 1987). Subsequent acid or enzymatic hydrolysis of the pentosan, which is primarily acetylxylan, produces a mixture of sugars, mostly D-xylose, some uronic acids and a significant quantity of acetic acid. The hydrolysates also contain sugar-degradation products such as furfural and hydroxymethylfurfural and a variety of aromatic lignin-degradation products that are generated during the steam-pretreatment and acid hydrolysis. These compounds, together with acetic acid, act synergistically to inhibit the fermentation activity of both bacteria and fungi (Ando et al. 1986; Beck 1986a; Nishikawa et al. 1988; Puls et al. 1985; Tran and Chambers 1986). The concentration of these water-soluble inhibitors in the hemicellulose hydrolysates makes fermentation to ethanol or other solvents more difficult.

The success of fermentation of the xylose in total hydrolysates of lignocellulosic substrates has been variable (Skoog and Hahn-Hagerdal 1988). Reports have appeared dealing with ethanolic fermentation of hemicellulose hydrolysates from various lignocellulosics (Liu et al. 1988; Tran and Chambers 1986; Beck 1986b; van Zyl et al. 1988; Watson et al. 1984), but none has dealt specifically with aspenwood hemicellulose.

In our attempts to improve the ethanolic fermentation of our steam-pretreated aspenwood hemicellulose by the yeast, *Pichia* stipitis CBS 5776, we tested both the combined enzymatic hydrolysis and fermentation (CHF) and separate hydrolysis and fermentation (SHF) approaches. We also studied the effectiveness of rotoevaporation and ethyl acetate extraction (Clark and Mackie 1984) on removing the fermentation inhibitors in an effort to determine the minimum pre-fermentation treatment of our hemicellulose hydrolysates if they are to be fermented separately from the cellulose hydrolysate. Separate hemicellulose fermentation is desirable to reduce the inhibition (diauxie) of xylose fermentation caused by excess glucose (duPreez et al. 1986).

MATERIALS AND METHODS

Strain and Culture Conditions

Pichia stipitis CBS 5776 was obtained from the National Research Council of Canada (NRCC) culture collection as NRC 2893. Cultures were maintained on YPX slants containing 1% w/v yeast extract, 2% peptone, 2% xylose and 2% agar. Inocula were grown in YMPX broth containing 0.3% yeast extract, 0.3% malt extract, 0.5% peptone and 5.0% xylose for 48 h at 30°C and 150 rpm. Cells were harvested at 9800 g for 10 m and washed once in 0.1% peptone water prior to inoculation. Inoculum cell density was 6.4 g dry wt L^{-1}.

Substrate preparation

Aspenwood chips were pretreated with saturated steam at 240°C for 80 s and water-extracted to obtain a dilute hemicellulose fraction as described previously (Brownell and Saddler 1987). The water-solubles were concentrated to 20% w/v solids by rotoevaporation at 55°C and adjusted to pH 5.0 with NH_4OH prior to hydrolysis.

Enzymatic hydrolysis

A hemicellulase preparation from Trichoderma harzianum E58 culture filtrate was used for enzymatic hydrolysis (Tan et al. 1987). Concentrated, steam-pretreated aspenwood water-solubles (14.6% w/v solids) were hydrolyzed at 45°C, pH 4.8, using 5000 xylanase units g^{-1} pentosan. Hydrolysis was stopped after 48 h by boiling the hydrolyzate for 15 m.

Acid hydrolysis

Water-solubles (20% w/v) were mixed with 3% w/v concentrated sulphuric acid and autoclaved for 1 h at 121°C. The resulting hydrolysate was filtered through Whatman No.1 paper.

Inhibitor removal treatments

Hydrolysates were rotoevaporated under vacuum to near dryness at 55°C to remove acetic acid. Lignin-degradation products were removed by 4 ethyl acetate extractions (1:1) of the hydrolysates, followed by rotoevaporation to remove the residual solvent.

Combined hydrolysis and fermentation (CHF)

Water-solubles were filter sterilized and diluted to either 2.5% or 5% w/v total solids with sterile fermentation medium containing 0.3% yeast extract and 0.2% diammonium phosphate. CHF was initiated by simultaneous addition of xylanase and inoculum at the concentrations described above. CHF was carried out at 30°C in

250 ml foam-stoppered flasks at 150 rpm. Total reaction volume was 100 ml.

Separate hydrolysis and fermentation

The same fermentation medium and conditions were used as described for CHF except that the water-solubles were previously acid or enzymatically hydrolyzed as described above. All fermentations were done in duplicate and average results reported. Control fermentations of pure xylose and glucose were also run.

Analytical

Acetic acid, ethanol, cellobiose, glucose, xylobiose and xylose were analysed by HPLC (Schwald and Saddler 1988). Sugar- and lignin-degradation products were analysed by HPLC (Burtscher et al. 1987). Dry weight was determined after samples were dried at 105°C for 24 h.

RESULTS AND DISCUSSION

Combined enzymatic hydrolysis and fermentation (CHF)

CHF normally offers the advantage of improving enzymatic hydrolysis efficiency by eliminating product inhibition of xylanase through concomitant xylose fermentation. However, deacetylation of the acetylxylan also occurs during enzymatic hydrolysis, generating acetic acid (Biely et al. 1986). The CHF results obtained during 72 h at 30°C are shown in Table 1. The

Insert Table 1

observed increase in xylobiose after 8 h, followed by its hydrolysis to xylose indicated both xylanase and xylosidase activities were present during CHF of both hemicelluose concentrations. The hydrolysis of cellobiose to glucose, apparent in the 5% w/v hemicellulose substrate, but not in the control, indicated that β-glucosidase activity was associated with *P. stipitis* and not the *Trichoderma harzianum* E58 hemicellulase preparation.

At both concentrations of hemicellulose tested, despite rotoevaporation treatment prior to CHF, the initial acetic acid was at least 2 g L^{-1}, which is sufficient to inhibit xylose fermentation by *Pichia stipitis* (van Zyl et al. 1988; Parekh et al. 1987). Cellobiose and glucose fermentation occurred at the 2.5% w/v total solids hemicellulose concentration. For xylose fermentation, however, the CHF approach was unsuccessful at both concentrations of hemicellulose tested. These results identified the need to separately hydrolyze the hemicellulose, using either acid or enzymes, so that the acetic acid generated could be removed prior to fermentation.

Separate hydrolysis and fermentation (SHF)

The SHF results, which compared the fermentability of acid and enzyme hydrolysates before and after inhibitor removal treatments, are summarized in Table 2. Both hydrolysates were fermentable at a concentration of 2.5% w/v total solids. Above 2.5% w/v total solids, the concentration of acetic acid and other inhibitors was apparently sufficient to prevent fermentation.

Insert Table 2

Both inhibitor removal treatments were capable of improving fermentability of the 5% w/v hydrolysates. The low initial concentrations of glucose were totally fermented within the first 24 h. The inhibitors that remained following the treatments still significantly decreased the xylose fermentation rates compared to the controls. Analysis of the hydrolysates before and after treatment indicated that furfural and several lignin-degradation products, some of which were identified and quantified, were present in both acid and enzyme hydrolysates. The acid hydrolysate contained significantly higher furfural, indicating that some degradation of pentose sugars had occurred in addition to that resulting from the steam-pretreatment. The presence of vanillin probably caused some inhibition as Tran and Chambers (1986) reported that 0.09 g L^{-1} vanillin caused over 50% reduction in ethanol yield from 40 g L^{-1} xylose by P. stipitis CBS 5776. However, even in the presence of 0.21 g L^{-1} vanillin and other inhibitors, we observed 77% utilization of the xylose in the rotoevaporated enzymatic hydrolysate. Tran and Chambers (1986) also reported that 1.3 g L^{-1} furfural had little effect on fermentation. For this reason, the low levels of furfural reported here were not considered inhibitory. The separate inhibitory effect of 0.8-1.0 g L^{-1} p-hydroxybenzoic acid was not determined; however, 1 g L^{-1} has been shown to inhibit glucose fermentation by Saccharomyces cerevisiae (Ando et al. 1986). The synergistic effect of a combination of low levels of these known inhibitors, and other unidentified lignin-derived compounds can cause a greater inhibiton than when present individually at less than their inhibitory concentrations (Beck 1986a; Tran and Chambers 1986).

The lower ethanol yield from the rotoevaporated acid hydrolysate compared to the similarly treated enzymatic hydrolysate ($Y_{p/s}$ = 0.13 and 0.34 respectively) may be a result of using NH_4OH instead of $Ca(OH)_2$ to adjust the acid hydrolysate to pH 5.0 for fermentation as suggested by others (van Zyl et al. 1988). Other unidentified, non-volatile inhibitors created during the acid hydrolysis may also be responsible for the reduced fermentability.

Ethyl acetate extraction was more effective in removing the inhibitors than rotoevaporation alone, even though residual acetic acid levels were equal. Rotoevaporation removed furfural and the majority of acetic acid but, as expected, this treatment did not reduce lignin-derivative levels. Ethyl acetate extraction removed all of the inhibitory compounds except for the residual acetic acid. This level of acetic acid (2.7 g L^{-1}) is sufficient to cause a slower fermentation (i.e., 60-72 h). The control fermentations of similar levels of initial sugars, with no acetic acid present, were completed within 24 h, producing near theoretical ethanol yields and maximum volumetric ethanol production rates (Q_p) almost triple those observed where acetic acid was present.

The fermentation profile for the 5% ethyl acetate extracted acid hydrolysate showed that the fermentation was still incomplete after 72 h (Fig. 1). An 8 h diauxic lag in xylose fermentation was observed as the 1.6 g L^{-1} initial glucose was fermented (data not shown). Xylose uptake rate was estimated from Figure 1 to be 0.24 g $L^{-1}h^{-1}$.

Insert Figs. 1 and 2 here

The 5% ethyl acetate extracted enzymatic hydrolysate fermentation profile showed that all fermentable sugars were consumed before 72 h (probably closer to 60 h if we interpolate between sample points) (Fig. 2). Both xylose and xylobiose were fermented concurrently after an 8 h diauxic lag caused by 0.8 g L^{-1} glucose initially present (data not shown). Xylose uptake rate estimated from Figure 2 (0.32 g $L^{-1}h^{-1}$) was 2.7 times that of xylobiose (0.12 g $L^{-1}h^{-1}$) during the first 40 h, but the uptake rates became near equal after 40 h when both xylose and xylobiose concentrations were 3.6 g L^{-1}.

The 1.8 g L^{-1} cellobiose present in the hemicellulose was probably a contaminant from the cellulose water-extraction step or the hemicellulase enzyme preparation. Cellobiose fermentation occurred after 40 h at which point both xylose and xylobiose concentrations had been reduced to 3.6 g L^{-1} (Fig. 2).

This is the first report of xylobiose fermentation by P. stipitis. Xylobiose has been shown to be an intracellular inducer of endo-1,4-β-xylanase in the yeast Cryptococcus albidus (Biely et al. 1980). P. stipitis CBS 5776 has been shown to produce low levels of endo-1,4-β-xylanase activity when grown on xylan (Lee at al. 1986). The ability to ferment xylobiose indicates that P.

stipitis produces an intracellular β-xylosidase activity similar to that reported for C. albidus (Biely et al. 1980).

CONCLUSIONS

The results of this study confirm that P. stipitis CBS 5776 is capable of fermenting the hemicellulose hydrolysates of steam-pretreated aspenwood to produce theoretical ethanol yields, provided the hydrolysates are suitably treated to remove fermentation inhibitors. Ethyl acetate extraction was more effective than rotoevaporation alone for reducing fermentation inhibition. Enzymatic hydrolysis of the hemicellulose produced less inhibitors than acid hydrolysis and allowed a faster fermentation to theoretical ethanol yields. Despite incomplete enzymatic hemicellulose hydrolysis, the xylobiose in the hydrolysate was fermented by P. stipitis concurrently with xylose, although at a reduced rate.

Our results with aspenwood support the conclusion that the wood pretreatment and inhibitor removal methods used in preparing hemicellulose hydrolysates should be capable of minimizing the level of residual acetic acid and lignin-degradation products. This is especially important if higher concentrations of hemicellulose hydrolysates are to be fermented by P. stipitis to produce economically recoverable ethanol concentrations in the final beer.

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TABLE 1. COMBINED ENZYMATIC HYDROLYSIS AND FERMENTATION (CHF) OF STEAM-PRETREATED ASPENWOOD HEMICELLULOSE (a)

SUBSTRATE (total solids) (% w/v)	TIME (h)	CELLOBIOSE (g/L)	GLUCOSE (g/L)	XYLOBIOSE (g/L)	XYLOSE (g/L)	ETHANOL (g/L)	ACETIC ACID (g/L)
2.5	0	1.0	0.2	1.3	1.8	0.0	2.0
	8	0.6	0.3	5.8	4.0	0.2	2.4
	24	0.4	0.4	5.0	5.4	0.3	2.6
	40	0.3	0.4	4.6	6.3	0.3	2.6
	72	0.2	0.0	3.5	6.3	0.6	2.6
5.0	0	1.6	0.3	1.9	3.7	0.0	4.0
	8	1.2	0.7	11.4	9.4	0.0	4.6
	24	0.9	0.9	9.3	12.7	0.0	5.3
	40	0.5	1.0	7.7	13.9	0.0	5.4
	72	0.3	1.1	6.1	16.0	0.0	5.3
CONTROL (No Yeast) 5.0	72	1.8	1.1	6.0	16.0	0.0	5.4

(a) see Materials and Methods for details

TABLE 2. CHARACTERIZATION AND FERMENTATION OF TREATED HEMICELLULOSE HYDROLYSATES FROM STEAM PRETREATED ASPENWOOD BEFORE AND AFTER INHIBITOR REMOVAL TREATMENTS

HYDROLYSATE		INHIBITOR REMOVAL	INITIAL TOTAL	INITIAL TOTAL	ACETIC ACID	FURFURAL (g/L)	p-HYDROXY-BENZOIC	VANILLIN (g/L)	FERMENTATION RESULTS (c)			
TYPE	%(w/v)	TREATMENT (a)	XYLOSE (g/L)(b)	GLUCOSE (g/L)	(g/L)		ACID (g/L)		PENTOSE USED(%)	Yp/s (d)	Qp (e)	MAX. ETHANOL (g/L)
ACID	2.5	NONE	12.3	0.9	3.1	0.14	0.53	0.10	100	0.41	0.09	5.4
ACID	5.0	NONE	23.7	1.7	6.1	0.28	1.07	0.21	0	0	0	0
ACID	5.0	EVAPORATION	21.8	1.8	2.8	0	1.04	0.15	17	0.13	0.07	3.1
ACID	5.0	EXTRACTION	19.3	1.6	2.7	0	0	0	93	0.41	0.13	8.5
ENZYME	2.5	NONE	10.5	1.4	2.8	0.02	0.41	0.12	82	0.29	0.03	3.4
ENZYME	5.0	NONE	20.2	2.6	5.3	0.05	0.82	0.23	0	0	0	0
ENZYME	5.0	EVAPORATION	21.0	2.8	2.7	0.01	0.81	0.21	77	0.34	0.08	8.0
ENZYME	5.0	EXTRACTION	20.5	2.7	2.7	0	0	0	100	0.47	0.20	11.0
PURE SUGAR CONTROLS:		NONE	11.8	2.8	0	0	0	0	100	0.44	0.27	6.4
		NONE	25.2	5.6	0	0	0	0	100	0.44	0.57	13.6

(a) Inhibitor removal treatments: Evaporation - rotoevaporation under vacuum at 55°C
Extraction - ethyl acetate extraction followed by rotoevaporation (see text)

(b) Expressed as xylose equivalents. Xylose was only pentose detected in acid hydrolysate
Initial xylose:xylobiose ratio was 1.8:1 in enzymatic hydrolysate

(c) Control results after 24 h; acid and enzyme hydrolysate results after 72 h

(d) Ethanol yield (g ethanol/g total fermentable substrates)

(e) Maximum volumetric ethanol production rate (g ethanol/L/h)

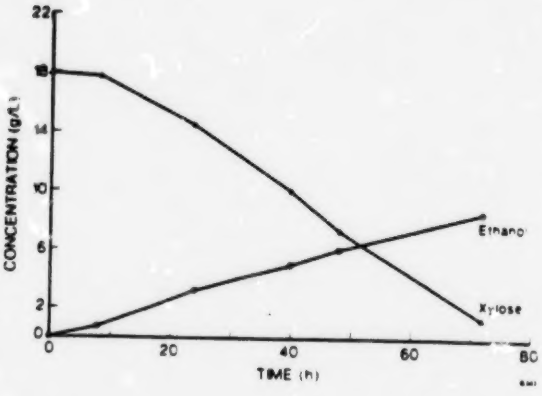

Fig. 1. Fermentation of 5% w/v steam-pretreated aspenwood hemicellulose *acid* hydrolysate by *Pichia stipitis* after ethyl acetate extraction to remove fermentation inhibitors.

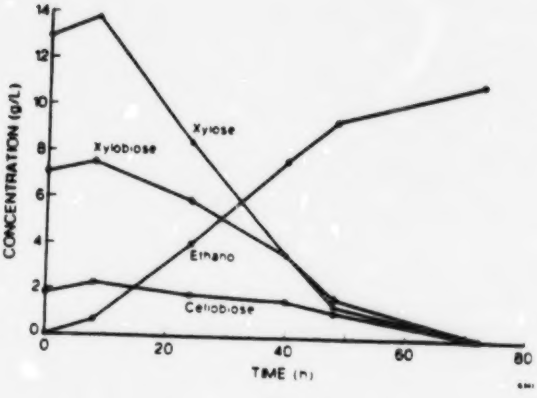

Fig. 2. Fermentation of 5% w/v steam-pretreated aspenwood hemicellulose enzymatic hydrolysate by Pichia stipitis after ethyl acetate extraction to remove fermentation inhibitors.

EFFECT OF INITIAL GLUCOSE:XYLOSE RATIO AND TOTAL SUBSTRATE CONCENTRATION ON XYLOSE FERMENTATION BY *PICHIA STIPITIS* CBS 5776

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Forintek Canada Corp.

ABSTRACT

To increase the yield of ethanol from steamed-pretreated aspenwood hemicellulose hydrolysates, the effect of initial glucose:xylose ratio (G:X) and total initial substrate concentration (S_0) on ethanol yield from xylose ($Y_{p/x}$) was studied. Both pure sugar mixtures and hemicellulose hydrolysates were fermented by *Pichia stipitis* CBS 5776 with S_0 ranging from 32 to 140 g/L and G:X between 0:1 and 2.2:1. For pure sugar fermentations, near theoretical $Y_{p/x}$ values of 0.44 to 0.46 g ethanol/g xylose supplied were obtained after 24 h from a S_0 of 50 g/L and G:X ratio of 1:4 or 1:1. $Y_{p/x}$ from hemicellulose hydrolysates were highest for $S_0 < 35$ g/L and G:X ratios near 1:4. Total xylose utilization was observed at higher S_0 and G:X ratios , but $Y_{p/x}$ values were greatly reduced. These results suggest that when combining hemicellulose and cellulose hydrolysates from steam pretreated wood as a strategy for diluting the fermentation inhibitors associated with hemicellulose and increasing S_0, the resultant G:X ratio and S_0 must be kept low enough to maximize ethanol production from xylose.

Dans le but d'accroître le rendement d'éthanol obtenu à partir d'hydrolysats d'hémicellulose de tremble pré-traités à la vapeur, on a étudié l'effet du rapport initial glucose:xylose (G:X) et de la concentration totale de substrat (S_0) sur le rendement de l'éthanol produit à partier du xylose ($Y_{p/x}$). Des mélanges de sucres purs et des hydrolysats d'hémicullulose ont été fermentés par *Pichia stipitis* CBS 5776, la valeur de S_0 variant de 32 à 140 g/L et le rapport G:X étant compris entre 0:1 et 2.2:1. Dans le cas des fermentations du sucre pur, on a obtenu un $Y_{p/x}$ approchant de la valeur théorique, soit de 0,44 à 0,46 g d'éthanol/g de xylose fourni, après 24 heures, la valeur de S_0 étant de 50 g/L et le rapport G:X étant de 1:4 au départ. La valeur obtenue pour $Y_{p/x}$ avec les hydrolysats d'hémicellulose était maximale lorsque $S_0 < 35$ g/L, les rapports G:X sont approximativement de 1:4. Lorsque S_0 et les rapports G:X étaient plus élevés, le xylose était totalement utilisé, mais les valeurs de $Y_{p/x}$ diminuaient de beaucoup. Ces résultats montrent que pour combiner des hydrolysats d'hémicellulose avec des hydrolysats de cellulose afin d'augmenter S_0 et de diluer les inhibiteurs de la fermentation associés à l'hémicellulose, il faut maintenir faibles le rapport G:X et S_0, ce qui permet de maximiser la production d'éthanol à partir du xylose.

INTRODUCTION

For the economic production of ethanol from wood hydrolysates it is essential that the hemicellulosic sugars, in addition to the hydrolysed cellulose, be efficiently fermented. Separation of both hemicellulose and lignin from the cellulose following steam-pretreatment of aspenwood has been shown to benefit subsequent enzymatic cellulose hydrolysis[1]. In addition, the presence of lignin in steam-pretreated aspenwood has been shown to reduce enzymatic cellulose hydrolysis efficiency by adsorbing cellulases[2]. Consequently, the separation of steam-pretreated wood into its three major fractions, cellulose, hemicellulose and lignin, prior to enzymatic hydrolysis and fermentation is required to improve the utilization efficiency of the total pretreated wood[3].

When aspenwood is fractionated following SO_2-catalyzed steam-pretreatment, the water-soluble hemicellulose fraction, which is primarily hydrolysed acetylxylan, contains a mixture of sugars, mostly D-xylose, with some uronic acids and a significant quantity of acetic acid. The hydrolysates also contain sugar-degradation products such as furfural and hydroxymethylfurfural and a variety of aromatic lignin-degradation products that are generated during the steam-pretreatment. These sugar- and lignin-degradation products, together with acetic acid, act synergistically to inhibit the fermentation activity of both bacteria and fungi[4,5,6,7,8,9]. The concentration of these water-soluble inhibitors in the hemicellulose hydrolysates makes fermentation to ethanol or other solvents more difficult.

The success of fermentation of the xylose in total hydrolysates of lignocellulosic substrates has been variable[10]. In our attempts to improve the ethanolic fermentation of our steam-pretreated aspenwood hemicellulose by the yeast, *Pichia stipitis* CBS 5776, we have found that rotoevaporation and ethyl acetate extraction treatments were able to remove the fermentation inhibitors from hemicellulose hydrolysates[9]. While rotoevaporation alone or steam-stripping[5,11] may be useful in removing acetic acid to non-inhibitory levels, the non-volatile inhibitors remain.

Intermittent feeding of cellulose hydrolysate to hardwood hemicellulose hydrolysate was shown to dilute inhibitors and improve ethanol yields using *Pachysolen tannophilus* when used with substrate containing <30 g/L total sugars[12]. However, the presence of excess glucose can repress xylose utilization[13].

In this study we have determined the inhibitory concentration of acetic acid on the fermentation of a mixed glucose and xylose substrate and the effect of the initial glucose:xylose (G:X) ratio and initial fermentable sugar concentration on the efficiency of xylose fermentation to ethanol. These results were part of a preliminary study to determine the feasibility of diluting the level of non-volatile inhibitors in hemicellulose hydrolysates with a cellulose hydrolysate (i.e. glucose) in order to both improve total fermentability and to increase the total fermentable sugars in the substrate for higher final ethanol concentration.

MATERIALS AND METHODS

Strain and Culture Conditions

Pichia stipitis CBS 5776 was obtained from the National Research Council of Canada (NRCC) culture collection as NRC 2893. Cultures were maintained on YPX slants containing 1% w/v yeast extract, 2% peptone, 2% xylose and 2% agar. Inocula were grown at pH 5.0 in YMPX broth containing 0.3% yeast extract, 0.3% malt extract, 0.5% peptone and 5.0% xylose for 48 h at 30°C and 150 rpm. Cells were harvested at 2000 g for 10 m and washed once in 0.1% peptone water prior to inoculation. Initial cell density for fermentation was approximately 10 g/L dry wt.

Substrate preparation

Aspenwood hemicellulose hydrolysate was obtained as the water-solubles following water-extraction of aspenwood chips that were pretreated with SO_2 and saturated steam at 200 or 210°C for 80 or 100 s as described elsewhere[14]. The dilute water-solubles were concentrated by rotoevaporation at 55°C to remove acetic acid, diluted to the appropriate solids concentration and adjusted to pH 5.0 with NH_4OH prior to fermentation.

Fermentation

All fermentations were carried out at 30°C in 50 ml volumes in 125 ml flasks fitted with foam stoppers, agitated at 150 rpm to provide the low aeration required for xylose fermentation. Fermentation medium contained 3 g/L yeast extract, 2 g/L diammonium phosphate. Ammonium hydroxide was used to adjust the pH to 5.0. D-Glucose and D-xylose were added as required after separate sterilization. D-Glucose was added to hemicellulose hydrolysates to increase the initial glucose:xylose ratio of the substrate as required.

Analytical

Acetic acid, ethanol, glucose, xylose, glycerol, and xylitol were analyzed by HPLC[15]. Dry weight determinations were done at 105°C for 16 h.

Ethanol Yield Calculations

The ethanol produced from glucose (P_g) was estimated based on 90% of the theoretical yield ($Y_{p/g}$) of 0.51 g ethanol/g glucose being obtained after total glucose utilization had occurred. This 90% yield estimate was based on several control glucose fermentations done under the conditions used in this study. The ethanol produced from xylose (P_x) was then estimated by subtracting P_g from the maximum ethanol produced from the total fermentable sugars (P_s). Therefore, the ethanol yield from xylose,

$$Y_{p/x} = (P_s - P_g)/X_0 \ ,$$

where X_0 is the initial xylose (g) in the substrate. Similarly, the ethanol yield from the total supplied sugars,

$$Y_{p/s} = P_s/S_0 \ ,$$

where S_0 is the initial total fermentable sugars in the substrate.

RESULTS AND DISCUSSION

Fermentation Inhibition by Acetic Acid

To determine the inhibitory concentration of acetic acid on the fermentation of mixed sugar substrate, the ability of *Pichia stipitis* to ferment 50 g/L of a 1:1 mixture of xylose and glucose was tested in the presence of 0 to 8 g/L acetic acid at pH 5.5 (48 h results shown in Fig. 1). The fermentation results indicated that <1 g/L acetic acid was not inhibitory to either glucose or xylose fermentation, with $Y_{p/s}$ exceeding 93% of the theoretical yield of 24 g ethanol/L. Above 1 g/L acetic acid, significant inhibition of xylose fermentation was observed, while inhibition of glucose fermentation did not occur until the acetic acid concentration exceeded 2.5 g/L. At acetic acid concentrations above 2.5 g/L, glucose repression of xylose fermentation would also be in effect because glucose consumption was less than 100% after 48 h. Glucose fermentation occurred in the presence of 5 to 8 g/L acetic acid, although at a greatly reduced rate.

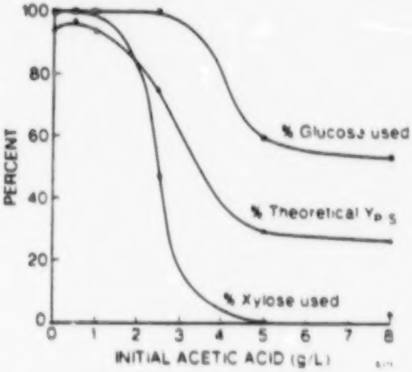

Figure 1. Acetic Acid Inhibition of Fermentation of 50 g/L of 1:1 Glucose:Xylose Mixture by *Pichia stipitis* After 48 h at pH 5.5

These results indicated that residual acetic acid in hemicellulose hydrolysates should be less than 1 g/L after rotoevaporation or steam-stripping for complete and rapid xylose fermentation. Similar results have been reported for inhibition of *Candida shehatae* xylose fermentation by acetic acid at 2.8 g /L, but not at 1.4 g/L at pH 5.5[3].

Effect of Glucose:Xylose Ratio and Total Fermentable Sugars on Xylose Fermentation

Despite treatments to remove acetic acid, the presence of non-volatile, lignin-derived inhibitors can still cause significant inhibition in hemicellulose hydrolysates containing 50 g/L total solids[9]. Because of this limitation we tested the feasibility of diluting hemicellulose hydrolysates with cellulose hydrolysate (i.e. glucose) to reduce the non-volatile inhibitor concentration while increasing the total fermentable sugars in the combined substrate. As a preliminary study, we determined the effect of increasing the glucose:xylose (G:X) ratio on the actual efficiency of xylose fermentation. Both pure sugar mixtures and glucose supplemented hemicellulose hydrolysates with G:X ratios of 0:1, 1:4 (similar to hemicellulose hydrolysates), 1:1 and 2.2:1 (similar to total wood hydrolysates) were tested at total fermentable sugar concentrations from 32 to 140 g/L (Table 1).

Pure Sugar Fermentations

From approximately 50 g/L total sugars, the theoretical ethanol yield from xylose ($Y_{p/x}$) of 0.46 g/g was obtained after 24 h using a substrate G:X ratio of 1:1. The other G:X ratios used also produced in excess of 86% of the theoretical $Y_{p/x}$ value after 24 h (Fig 2). As expected, xylose consumption rate decreased with increasing G:X ratio, because of glucose repression of xylose fermentation. Very little ethanol consumption by *P. stipitis* was noticed during the 24 h following depletion of the sugar substrate. Recently, the continuous fermentation of 47.5 g/L of 1:3.75 G:X ratio by a flocculent strain of *P. stipitis* was reported, producing 19 g/L ethanol, which represents an estimated $Y_{p/x}$ of 0.39[16].

Table 1. ETHANOL YIELDS FROM FERMENTATION OF DIFFERENT MIXTURES OF GLUCOSE AND XYLOSE BY *Pichia stipitis* CBS 5776

SUBSTRATE	GLU:XYL RATIO	TOTAL SUGARS (g/L)	ETHANOL (g/L)	$Y_{p/x}$ (a)	$Y_{p/s}$ (b)
PURE SUGARS	0:1	47	19.4	0.42	0.42
	0:1	77	30.6	0.40	0.40
	1:4	49	21.4	0.44	0.44
	1:4	78	31.1	0.39	0.40
	1:1	47	21.4	0.46	0.45
	1:1	79	32.1	0.37	0.41
	1:1	140	39.6	0.11	0.28
	2.2:1	47	20.6	0.42	0.44
	2.2:1	77	31.6	0.33	0.41
HEMICELLULOSE (c) HYDROLYSATES					
(210°C/80s)	1:5	37	12.7	0.32	0.34
SAME + GLUCOSE	1:1	64	22.4	0.25	0.35
SAME + GLUCOSE	2.4:1	135	39.0	0.00	0.29
(200°C/100s)	1:4	32	13.4	0.41	0.42
SAME + GLUCOSE	1:1	54	21.6	0.34	0.40
SAME + GLUCOSE	1.6:1	66	23.0	0.19	0.35

(a) Yield of ethanol from xylose, estimated based on 90% conversion of glucose present to ethanol.

(b) Yield of ethanol from total glucose and xylose in substrate.

(c) Water solubles from SO_2-pretreated aspenwood steamed for the indicated time and temperature. Hydrolysates were treated to reduce acetic acid to <1 g/L before fermentation.

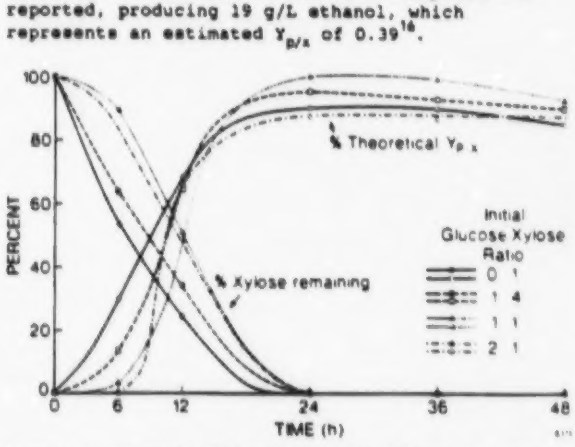

Figure 2. Ethanol Production from Xylose in 50 g/L Pure Sugar Substrates with Various Glucose:Xylose Ratios

From approximately 80 g/L total sugars, the theoretical ethanol yield from xylose was significantly decreased with all G:X ratios used (Fig 3). The G:X ratios of 0:1 and 1:4 produced around 84% of the theoretical $Y_{p/x}$ after a fermentation time of 36 h. Total xylose utilization was observed after 48 h using G:X ratios of 1:1 and 2.2:1, however the respective $Y_{p/x}$ values dropped to 0.37 and 0.33 g/g, or 80% and 70% of the theoretical value. The effect of increasing the total sugars was more pronounced when 140 g/L of 1:1 G:X ratio was fermented (Table 1). Total xylose and glucose utilization was observed after 72 h, however $Y_{p/x}$ was only 0.11 g/g or 24% of theoretical and $Y_{p/s}$ was only 0.28 g/g. Similar low $Y_{p/s}$ values of 0.31 and 0.27 g/g can be calculated from recently reported fermentation data for 145 g/L total sugars and G:X ratio of 1:1 using *P. stipitis* and *Candida shehatae* respectively, even though glucose addition was delayed for 24 h[13].

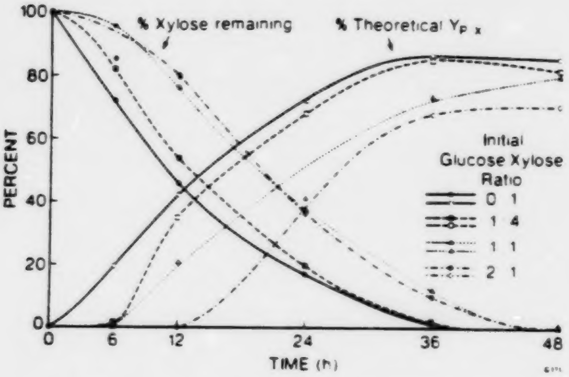

Figure 3. Ethanol Production from Xylose in 80 g/L Pure Sugar Substrates with Various Glucose:Xylose Ratios

The results of the pure sugar studies suggested that using cellulose hydrolysate to dilute the hemicellulose hydrolysate should be done such that the initial fermentable sugars are around 50 g/L with a G:X ratio of approximately 1:1. These findings were then applied to fermentation of actual hemicellulose hydrolysates.

Hemicellulose Hydrolysate Fermentations

Two different aspenwood hemicellulose hydrolysates were tested. The hydrolysates were obtained by water-extracting aspenwood chips following the SO_2-catalyzed steam-pretreatments described in Table 1. Both hydrolysates were rotoevaporated to 20% total solids, at which point acetic acid concentration was <1 g/L. Each was then diluted with either water or glucose solution to obtain different G:X ratios with the same initial xylose concentration (equivalent to that present in 5% w/v total solids for each hydrolysate).

The 210°C/80s hemicellulose hydrolysate had an initial G:X ratio of 1:5. Total xylose fermentation occurred after 36 h, however $Y_{p/x}$ was only 0.32 g/g or 70% of theoretical. A 4 g/L cell dry wt increase and 0.85 g/L xylitol were also produced. Increasing the G:X ratio to 1:1 with glucose also increased the total sugars to 64 g/L. While 96% of the xylose was used in 36 h and the final ethanol concentration increased, the estimated $Y_{p/x}$ decreased to 0.25 g/g or 54% of theoretical. Increasing the G:X ratio to 2.4:1 and the total sugars to 135 g/L raised the final ethanol concentration to nearly 4% (w/v). Total xylose utilization was observed after 72 h, however none of the ethanol could be attributable to xylose fermentation, by our estimation method, and $Y_{p/s}$ was only 0.29 g/g. A 7.5 g/L cell dry wt increase and 2.7 g/L xylitol were also produced.

The 200°C/100s hemicellulose hydrolysate had an initial G:X ratio of 1:4 was more fermentable than the other hydrolysate producing a $Y_{p/x}$ of 0.41 g/g (89% of theoretical) and total xylose utilization after 48 h. Increasing the G:X ratio to 1:1 and the total sugars to 54 g/L did not improve the fermentation yields and were significantly less than the comparable pure sugar results, taking 96 h to complete compared to 24 h to complete. Increasing the G:X ratio to 1.6:1 to further dilute this hydrolysate did not produce any improvement in xylose fermentation rate or yield. These results suggest that further dilution of the non-volatile inhibitors was necessary. However, further dilution with glucose would increase the total sugars and G:X ratio to where $Y_{p/x}$ values near zero would be expected, based on the pure sugar studies discussed above.

CONCLUSIONS

These studies have shown that fermentation of SO_2-catalyzed steam-pretreated aspenwood hemicellulose hydrolysates at concentrations of 5 wt% total solids and above cannot be improved simply by dilution with glucose (i.e. cellulose hydrolysates). Despite the reduction of acetic acid to <1 g/L prior to fermentation, the non-volatile inhibitors that remain apparently require dilution so great as to make the G:X ratio and total sugar concentrations too high for efficient xylose fermentation as shown by the pure sugar fermentations.

Further studies are needed to better define the optimum steam-pretreatment conditions that would minimize lignin-degradation products in the hemicellulose hydrolysate or to develop a cost-effective inhibitor removal treatment that would permit efficient xylose fermentation in hydrolysates containing greater than 35 g/L total sugars.

ACKNOWLEDGEMENT

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VI. MARKET STUDY FOR ETHANOL, BUTANOL AND BUTANEDIOL

All values in Canadian $, 1986

An integral part of the feasibility study for the utilization of the hemicellulose component was the assessment of the markets for ethanol, butanol and butanediol. This market study should help to focus the research towards the most profitable process products.

The Canadian ethanol market is influenced to a certain degree by the ethanol market in the United States. In the USA there is currently a balance between supply and demand and future growth is projected at a steady 8.5% increase per year. The current price for fuel grade ethanol is between $0.54 - 0.62 per liter. The United States has imposed an import duty on ethanol of $0.145/liter. In the USA, ethanol is blended with gasoline to produce gasohol which has a 5.2% market share of the 386 billion liter/annum gas market.

In Canada there exists a demand/supply imbalance and 32 million liters of industrial ethanol are exported to the USA annually. The demand for ethanol in Canada is 44 million liters/annum whereas the production capacity is 129 million liters/annum. Based solely on these figures it would make little economic sense to increase the Canadian capacity to produce industrial ethanol. There are, however, two areas where an expansion of ethanol's market volume can be expected; the automotive fuel sector and the chemical industry. Von Bremen and Scholmtzi (1986) have recognized several areas in the automotive fuel sector where ethanol can be used in larger quantities. These areas are:

1. as a fuel without additional crude oil components (altered motor technology is needed);
2. as a fuel component which substitutes for only part of the crude oil-based gasoline;
3. for synthesizing octane enhancing fuel components which could substitute for other octane boosters in gasoline production;
4. as a co-solvent in methanol containing gasoline;
5. as a diesel oil substitute (as plain ethanol or admixed to diesel oil);
6. for transformation to ethanol derivatives to be used as diesel oil substitutes.

The expansion of the ethanol market into the chemical industry via formation of ethylene is difficult to assess because of the complexity of this industry and the competition from both methanol and gas oil. Ethanol's full potential will be realized with further technological breakthroughs on both the production of ethanol and its efficient use.

The butanol market situation is similar to the ethanol situation in that a large potential market exists for butanol. This will depend on the automotive fuel sector where butanol can be used as a co-solvent in methanol containing gasoline. At present, there is an overcapacity of industrial grade butanol in both Canada and the USA and butanol production from biomass will be feasible only if automotive fuels shift to methanol blends.

The market for butanediol derived from fermentation of biomass has received little attention due to the fact that the butanediol produced from fermentation is 2,3-butanediol and the traditional intermediate feedstock in the petrochemical industry is 1,4-butanediol. There are, however, markets for derivatives of 2,3-butanediol. Two organic intermediates that can be derived from 2,3-butanediol and are traded in large quantities are methyl ethyl ketone, and 1,3-butadiene.

Prices for methyl ethyl ketone have fluctuated from $1.08 to $1.32/kg for the past four years. The Canadian demand in 1985 was 10.7 kt but demand has been flat since 1980. The major end use of this chemical is in specialty coatings, vinyl fabrics, and adhesives.

The price for 1,3-butadiene is $1.13/kg. Polysar is the sole Canadian manufacturer producing 160 Kt/year. Butadiene is produced at Polysar as a by-product of ethylene manufacture. Polysar is a net importer of butadiene and can export 70 Kt/year of butadiene. Higher production levels of butadiene are at the moment tied to the ethylene market as ethylene is the primary product of the process.

The most likely end use of 2,3-butanediol would appear to be butadiene as the market is large and there exists a distinct possibility for expansion and for export. Since current manufacture is as a co-product of ethylene production, market supply and price will depend on ethylene capacity. The process for the conversion of 2,3-butanediol to butadiene should be further investigated in order to determine the economic feasibility of this extra step.

All of the chemicals produced from this biomass process are linked to the petrochemical industry, thus the price and market fluctuations for fermentation derived chemicals will depend on the price and availability of oil. There are political and environmental considerations in the production of liquid fuels from Canadian resources and these considerations may favour the expansion of ethanol and butanol in the automotive fuel sector, economics notwithstanding.

The issue of the use of ethanol in gasoline was addressed by a Parliamentary Committee (1986) that looked at the broader issue of employing a variety of fuel oxygenates (alcohols or ethers) in Canadian gasoline stocks. The committee made three recommendations listed below:

1. The Committee recommends the use of methanol and ethanol as blending agents in Canadian gasoline.

2. The Committee recommends that the Federal Government more extensively support research, development, and demonstration required to introduce alcohol-gasoline blends on a broad basis. This support should include new

approaches to fuel alcohol utilization and production - including the derivation of ethanol from sources of starch, sugar, and cellulose - which could remove economic or technical constraints to alcohol-gasoline blending.

3. The Committee recommends that the Federal Government, in cooperation with the provincial governments and industry, establish guidelines and standards for alcohol-blended gasoline.

This study indicated that we should focus our efforts on production of ethanol and thus research in the production of butanol and butanediol was phased out.

VII. DISCUSSION

Cellulose hydrolysis

Cellulose hydrolysis by cellulase was characterised by an initial rapid rate, which levels off quite drastically after about 8 hours. The initial rate and the extent of hydrolysis was strongly dependent on the susceptibility of the substrate, and the composition and concentration of the cellulase system. The decline in the hydrolytic rate throughout the reaction could be attributed to changes occurring either to the enzymes or to the substrate.

A number of factors affecting and limiting enzymatic hydrolysis of a model cellulose, Solka FLoc BW300, using *T. harzianum* E58 cellulase were investigated. A basic problem in cellulose hydrolysis was that the conditions employed for hydrolysis are not conducive to maintenance of enzyme activity or stability. High substrate concentration, high temperature, long incubation times, cellulase adsorption and end-product inhibition, all reduced the activity of the enzymes. Although cellulases were partly inactivated at 45°C, enzyme inactivation was not a major cause of the decline in hydrolysis. Site blockage of adsorptive or catalytic sites on the substrate by inactivated enzymes did not play a role in limiting hydrolysis.

As enzyme adsorption is a prerequisite for hydrolysis the relationship between the two processes were studied. The optimal conditions for hydrolysis lie within the pH and temperature range of optimal adsorption onto cellulase. As the concentration of enzyme present in the system was increased the amount of enzyme adsorbed to the substrate increased, up to a saturation point. Hydrolysis of the substrate to reducing sugars reflected this pattern and it was determined that the substrate had a limited number of binding sites for enzyme adsorption. Additional cellulase remained in solution and played only a minor role in hydrolysis.

The enzyme adsorbed to the substrate in the initial stage of hydrolysis played a strong role in governing the overall reaction. When working with an insoluble substrate it was the substrate or the adsorptive process that controlled the overall rate. The enzyme loading at which substrate saturation was reached was the most economical enzyme concentration for use in hydrolysis. Enzyme saturation could be determined by studying either the adsorption of the cellulase preparation onto the substrate or by employing a combined hydrolysis and fermentation process.

End-product inhibition of the cellulase and β-glucosidase by cellobiose and glucose was one of the major factors limiting hydrolysis. Inhibition could be alleviated by supplemental β-glucosidase or by removing the sugars produced by fermenting them to ethanol. Once the problem of end-product inhibition was removed, the major impediment to hydrolysis was the substrate. A faster rate of reaction could be obtained by increasing the susceptibility of the substrate to enzymatic attack.

Crystallinity and surface area accessible to the enzyme were investigated to observe the relationship between structural features of the substrate and its hydrolysis. A slight increase in crystallinity was found during a hydrolysis period of four days. The accessible surface area increased drastically in the initial phase of hydrolysis, then slowly declined through the remainder of hydrolysis. The substrate became more resistant to enzymatic attack during hydrolysis due to structural changes, and a 70% drop in accessibility of the substrate to hydrolysis was noted. This indicated that the substrate becomes more recalcitrant as hydrolysis proceeds and this was the major factor in the drastic decrease in the rate of hydrolysis.

The objective of a cellulose conversion process is to obtain high yields of glucose, using low enzyme loadings and short residence times. Our research indicated that the structure of the substrate primarily limited hydrolysis due to its insoluble and recalcitrant nature. Treatment of the substrate prior to, or during, hydrolysis would probably be a promising area on which to concentrate further research.

For a more complete discussion on the factors limiting hydrolysis the reader is directed towards the work of Hogan (1989).

Recycle

In cellulose hydrolysis there is the opportunity for two types of recycle; recycle of enzymes after the hydrolysis period and recycle of cellulose remaining after hydrolysis. Cellulose is mainly used for the production of glucose but a portion was diverted towards enzyme production, thus reducing the glucose yield attainable. A mass balance analysis of hydrolysis and enzyme production indicated three areas that would impact on glucose yield. These areas were enzyme yield, recycle of enzymes from the hydrolysis reactor and recycle of unhydrolysed cellulose from the hydrolysis reactor to the enzyme production reactor. The work in this portion of the project focused on increasing enzyme yield from *T. harzianum* and investigating the feasibility of two recycle schemes. This work was performed using pretreated aspenwood as the substrate.

Enzyme recycle

Only low levels of cellulase enzymes were recoverable from the filtrates of the hydrolysis mixture of steamed treated aspenwood. The residual material adsorbed or inhibited the cellulase enzymes so as to make them unsuitable for recycle. Lignin itself did not adsorb appreciable levels of enzyme, but in combination with small quantities of cellulose was able to adsorb significant quantities of cellulase.

Substrate recycle

The unhydrolysed substrate from the cellulose hydrolysis reactor was used as the growth substrate for enzyme production. This material could be used in combination with fresh cellulose to produce an active cellulase complex. The presence of high levels of lignin at this stage was found to inhibit the growth of the organism. The mass balance analysis indicated that this approach had a positive impact on the overall glucose yield.

Enzyme yield

A major problem with the use of *T. harzianum* E58 was the low enzyme productivity and yield. A large part of the project consisted of investigating ways of increasing productivity by optimizing fermentor conditions. This activity was not successful and we concluded that, in its present form, this organism is unsuitable for a commercial bioconversion process.

As an alternative to using cellulose for enzyme production we investigated the use of lactose for this purpose. This substrate is widely used for enzyme production with *T. reesei*. With *T. harzianum* the use of lactose as the substrate for enzyme production resulted in lower enzyme yield and quality. This knowledge led us to investigate the use of the residual cellulose (after hydrolysis) as the growth substrate. This proved to be a good substrate and demonstrated the effect that the physical characteristics of the cellulose had on the induction of the cellulase complex.

The research in recycle outlined the problems associated with lignin and the need to remove lignin prior to enzyme production and cellulose hydrolysis. Lignin was shown to be inhibitory to the enzyme production step and to the cellulase themselves, although it did not affect hydrolysis. Appreciable amounts of the cellulase enzymes adsorbed onto the lignin-cellulose complex, making them unavailable for recycle.

Hemicellulose Fermentation

Fermentation Inhibitors in Hemicellulose Hydrolysates

Acid or enzymatic hydrolysis of the water-soluble hemicellulose produced a mixture of sugars, mostly D-xylose, some uronic acids and a significant quantity of acetic acid. The hydrolysates also contained sugar-degradation products such as furfural and hydroxymethylfurfural and a variety of aromatic lignin-degradation products that were generated during the steam-pretreatment and acid hydrolysis. These compounds, together with acetic acid, act synergistically to inhibit the fermentation activity of both bacteria and fungi (Ando et al. 1986; Beck 1986; Nishikawa et al. 1988; Puls et al. 1985; Tran and Chambers 1986). The concentration of these inhibitors in the steam-pretreated aspenwood hemicellulose water-solubles (SEA-WS) made fermentation to ethanol or other solvents very difficult.

Butanol or Butanediol Production

Preliminary studies on SEA-WS fermentation were begun using *Klebsiella pneumoniae* to produce 2,3-butanediol and *Clostridium acetobutylicum* to produce butanol, acetone and ethanol. Both bacteria were capable of efficient xylose conversion when a pure sugar substrate was used, however the inhibitory nature of SEA-WS towards both of these fermentations caused poor utilization of the hemicellulose hydrolysates. Some of the lignin-derived inhibitors of the butanediol fermentation were identified and reported under a separate contract (Nishikawa *et al.*, 1988).

The Forintek market study indicated that ethanol was the best hemicellulose fermentation product with respect to potential market. Consequently, further work on butanediol or butanol fermentation was stopped in favour of ethanol production.

Ethanol Production

Combined enzymatic hydrolysis and fermentation (CHF) and separate hydrolysis and fermentation (SHF) approaches were tested as means of increasing ethanol yields from *Pichia stipitis*. We also studied the effectiveness of rotoevaporation and ethyl acetate extraction on removing the fermentation inhibitors. We concluded that *P. stipitis* was capable of fermenting the hemicellulose hydrolysates of steam-pretreated aspenwood to produce theoretical ethanol yields, provided the hydrolysates are suitably treated to remove fermentation inhibitors. Ethyl acetate extraction was more effective than rotoevaporation alone for reducing fermentation inhibition. Enzymatic hydrolysis of the hemicellulose produced less inhibitors than acid hydrolysis and allowed a faster fermentation to theoretical ethanol yields. During this study, the ability of *P. stipitis* to ferment xylobiose to ethanol was demonstrated for the first time. Despite incomplete enzymatic hemicellulose hydrolysis, the xylobiose in the hydrolysate was fermented by *P. stipitis* concurrently with xylose, although at a reduced rate.

Our results with aspenwood supported the conclusion that the steam pretreatment and inhibitor removal methods used in preparing hemicellulose hydrolysates should be capable of minimizing the level of residual acetic acid and lignin-degradation products. This is especially important if higher concentrations of hemicellulose hydrolysates are to be fermented by *P. stipitis* to produce economically recoverable ethanol concentrations in the final beer.

During this study, a separate Forintek study on acid-catalyzed steam pretreatments found that the use of SO_2 saturation of the wood chips prior to steaming produced a water-soluble fraction that contained totally hydrolyzed hemicellulose (SO_2-SEA-WS), eliminating the separate hydrolysis of SEA-WS

(Brownell *et al.*, 1989). This hemicellulose hydrolysate contained more glucose than SEA-WS due to partial cellulose hydrolysis. By varying the treatment time and steam temperature the ratio of glucose to xylose in the water-solubles could be changed. Because the acetylxylan in hemicellulose was totally hydrolysed, the acetic acid in the water-soluble fraction could be easily removed by an evaporation step prior to fermentation. The fermentability of these SO_2-SEA-WS substrates were tested in our study of the effect of glucose:xylose ratio on the efficiency of xylose fermentation by *P. stipitis*.

We determined the concentration of acetic acid that inhibited the fermentation of a mixed glucose and xylose substrate and the effect of the initial glucose:xylose ratio and initial sugar concentration on the efficiency of xylose fermentation to ethanol. This preliminary study determined the feasibility of diluting the level of non-volatile inhibitors in hemicellulose hydrolysates with a cellulose hydrolysate (i.e. glucose) in order to both improve hydrolysate fermentability and to increase the total fermentable sugars in the substrate to obtain higher final ethanol concentration for more economic product recovery.

The results indicated that acetic acid in 5% (w/v) hemicellulose hydrolysates (approx. 5-8 g/L) must be reduced to <1 g/L prior to fermentation to avoid inhibition of xylose fermentation. Inhibition of glucose fermentation occurred at acetic acid concentrations around 2.5 g/L.

At glucose to xylose ratios above 1:1 and total initial sugar concentrations greater than 50 g/L, the efficiency of xylose fermentation to ethanol was significantly reduced. Fermentation of SO_2-catalyzed steam-pretreated aspenwood hemicellulose hydrolysates at concentrations above 5 wt% total solids could not be improved simply by dilution with glucose (i.e. cellulose hydrolysate). Despite the reduction of acetic acid to <1 g/L prior to fermentation, the non-volatile inhibitors that remained in 5% (w/v) hemicellulose hydrolysates apparently required dilution so great as to make the glucose to xylose ratio and total initial sugar concentrations too high for efficient xylose fermentation.

Mass balance

The process design outlined in Figure 1 was used as a guide to incorporate the various parts of this project. Steamed aspenwood was processed using the process steps at a laboratory scale and the results extrapolated to a 500 OD tonnes/day plant (Figure 2). From a theoretical input of 500 tonnes of aspenwood, 482.5 tonnes of steam treated material could be recovered. This material required 10,875 tonnes of water to extract 707 tonnes of water soluble material. The water soluble material contained the equivalent of 53.5 tonnes of xylose and could be fermented to 17.3 tonnes of ethanol. The water insoluble residue (364.5 tonnes) when treated with 29.4 tonnes of caustic, produced 90.4 tonnes of soluble alkali material. From this, 79 tonnes of lignin could be recovered. The alkali insoluble residue (261 tonnes) was

Simplified Flow Diagram of the Process Development Unit

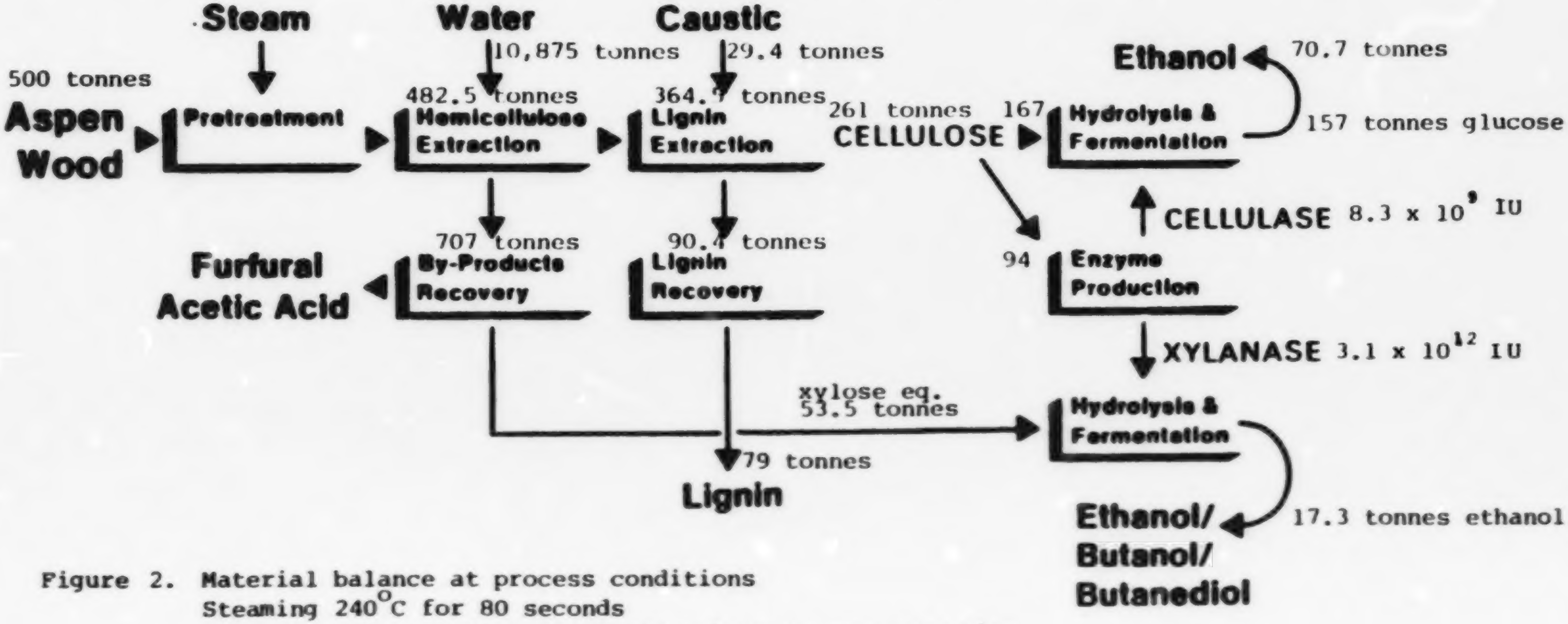
Steam
Water
Caustic
10,875 tonnes
29.4 tonnes
500 tonnes
Aspen Wood
Pretreatment
482.5 tonnes
Hemicellulose Extraction
364.9 tonnes
Lignin Extraction
261 tonnes
CELLULOSE
167
Hydrolysis & Fermentation
Ethanol
70.7 tonnes
157 tonnes glucose
CELLULASE 8.3 x 10⁹ IU
707 tonnes
By-Products Recovery
Furfural
Acetic Acid
90.4 tonnes
Lignin Recovery
94
Enzyme Production
XYLANASE 3.1 x 10¹² IU
xylose eq.
53.5 tonnes
Hydrolysis & Fermentation
79 tonnes
Lignin
Ethanol/
Butanol/
Butanediol
17.3 tonnes ethanol

Figure 2. Material balance at process conditions
Steaming 240°C for 80 seconds
Enzyme loading of 50 IU/g at 10% substrate concentration
Cellulase productivity 18 FP IU/L/h
100% hydrolysis in 4 days
88% of theoretical conversion of glucose to ethanol
73% of theoretical conversion of xylose to ethanol

predominantly cellulose and was diverted either towards enzyme production or cellulose hydrolysis. Ninety-four tonnes of material were required to produce enough cellulase enzymes to hydrolyse the remaining alkali insoluble residue. This material could be hydrolysed to 157 tonnes of glucose and subsequently fermented to 70.7 tonnes of ethanol. In total 88 tonnes of ethanol could be produced from the hemicellulose and cellulose component of 500 tonnes of aspenwood.

The material balance highlighted numerous problems with the overall process. The high volumes of water used for extraction purposes are unrealistic for process consideration and work on efficient extraction processes are needed for this process. The fermentation of the hemicellulose stream could only be performed at low substrate concentrations, again an unrealistic process parameter.

Probably the biggest problem with the process was the use of *T. harzianum* as the cellulase producer. The low enzyme productivity and yield of this organism required a large amount of cellulose to be diverted to enzyme production. This diversion decreased substantially the ethanol that could be derived from the process.

The mass balance clearly indicated that conditions used in this process design would not be economical and that the areas of extraction, inhibitor remmoval and enzyme productivity must be addressed before further efforts are directed towards a commercial process based on the conditions used in this project.

Although no economical process for the production of ethanol from aspenwood resulted from this project, major advances in the scientific knowledge base were made. A major goal of the project was to determine the feasibility of using the cellulase system of *T. harzianum* E58 in a commercial process. We demonstrated that in its present condition this organism was not suitable for use in a commercial bioconversion process.

The research demonstrated the interdependency among pretreatment, cellulose hydrolysis, hemicellulose fermentation and enzyme production. Much of this base knowledge is now being applied to other conversion processes and research is continuing towards a commercial process.

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Progress report
CFS # 04-53-12-402
March, 1989

SUMMARY

TITLE: Combined enzymatic hydrolysis and fermentation of aspenwood using enzymes derived from *Trichoderma harzianum* E58.

CONTRACTOR:
Forintek Canada Corp.
800 Montreal Road
Ottawa, Ontario
K1G 3Z5

SCIENTIFIC AUTHORITY:
R. D. Hayes
Energy Mines and Resources
460 O'Connor St.
Ottawa, K1A 0E4

PRINCIPAL INVESTIGATORS:
CELLULOSE CONVERSION: M. Mes-Hartree, C. Hogan and G. Louis-Seize
HEMICELLULOSE CONVERSION: J. Wilson, N. Nishikawa, L. Deschatelets and E. Yu

CONTRACT NO: 02SQ.23216-4-6391
COST: $713,886 Fiscal year: 85/86 $230,000
86/87 $282,167
87 $201,719

OBJECTIVE:
Investigate the conversion of aspenwood to ethanol, butanol or butanediol using an enzymatic hydrolysis approach based on the enzyme complex derived from *Trichoderma harzianum* E58.

RESULTS AND CONCLUSIONS:
Aspenwood was steam pretreated and then water and alkali extracted. This resulted in a water-soluble fraction containing hemicellulose, an alkali-soluble fraction containing lignin and an insoluble fraction that was 84% cellulose. The hemicellulosic fraction required hydrolysis prior to fermentation to ethanol by the organism *Pichia stipitis*. Inhibitors produced during the steaming process remained a problem throughout the investigation. Research on the production of butanol and butanediol was phased out during the course of the project because ethanol had the greater market potential.

The cellulose fraction of aspenwood was used for production of enzymes by *T. harzianum*. The enzymes produced hydrolysed the cellulose fraction to glucose, which was easily fermented to ethanol. The rate of hydrolysis was initially quite rapid but slowed considerably after 8 hours. This was found to be largely due to the structural limitations imposed by the physical characteristics of the cellulose. The need to divert cellulose to enzyme production decreased the potential ethanol yield obtainable from cellulose. Various ways of overcoming this problem were investigated but with limited success. We concluded that *T. harzianum* E58 in its present form was an unsuitable candidate for a commercial bioconversion process.

COMMENTS:
During this study, a separate Forintek study demonstrated that SO_2-catalysed steam pretreatment resulted in complete hydrolysis of the hemicellulose fraction, thus eliminating the need for a separate hydrolysis reaction. The technical achievements of this project have been reported in the scientific literature in the form of nine papers.